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Acknowledgment of reviewers/Translators
Remerciement aux évaluateurs et aux traducteurs

The continued success of the Journal is due in no small measure to the willingness of reviewers to assist the editors by their evaluation of manuscripts. Their generous efforts contribute both to the quality of the Journal and to the quality of veterinary research. Reviewers of manuscripts published, rejected, or expired from November 2019 to October 2020 are listed below. The Editors and Editorial Board wish to thank these colleagues for the donation of their time and the sharing of their expertise.

Abdul Careem, M. Faizal
Atkins, Clarke
Baby, Sibi
Barron, Heather
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Krell, Peter
Lawrence, Yuri
Lee-Fowler, Tekla
Lemeytger, Julie
Lennox, Angela
Liu, Frank
Lopeti, Lavulo

The Editors and Editorial board wish to also thank the following translators for their excellent service.

Luby, Chris
Mayer, Monique
Melzer, Falk
Messier, Serge
Moawad, Adel
Nemec Svetev, Alenka
Niu, Dangyan
Orcutt, Connie
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Timsit, Eduard
Trott, Darren
Weese, Scott
Wood, Darren
Yamashita, Kazuto
Zhang, Hui

Le succès continu de la Revue est attribuable en très grande partie à la collaboration des lecteurs qui appuient les rédacteurs en évaluant les manuscrits. Leurs généreux efforts contribuent tant à la qualité de la Revue qu’à celle de la recherche vétérinaire. Voici la liste des lecteurs des manuscrits qui ont été publiés ou rejetés pour la période de novembre 2019 à octobre 2020. Les rédacteurs et le Comité de la rédaction désirent remercier ces collègues du don de leur temps et du partage de leur expertise.

André Bisaillon • Serge Messier
Nasal bacterial microbiota during an outbreak of equine herpesvirus 1 at a farm in southern Ontario

Diego E. Gomez, Luis G. Arroyo, Brandon Lillie, J. Scott Weese

Abstract

The objective of this study was to investigate the nasal bacterial microbiota of healthy horses and horses infected with equine herpesvirus 1 (EHV-1). The nasal bacterial microbiota of 10 horses infected with EHV-1 and 11 control horses from a farm experiencing an outbreak was characterized using the Illumina MiSeq platform targeting the V4 region of the 16S ribosomal RNA gene. The nasal bacterial microbiota of healthy horses and EHV-1 horses was significantly different in community membership and structure. Horses shedding EHV-1 had lower bacterial richness ($P = 0.002$), evenness ($P = 0.008$), and diversity ($P = 0.026$) than healthy horses. Healthy horses had a higher relative abundance of Firmicutes and Bacteroidetes, but lower Proteobacteria than horses with EHV-1 ($P < 0.05$). This study provides the basis for generating hypotheses and investigations on the role of bacterial-viral interactions in the health and diseases of adult horses.

Résumé

L'objectif de cette étude était d'étudier le microbiote bactérien nasal de chevaux sains et de chevaux infectés par l'herpèsvirus équin 1 (EHV-1). Le microbiote bactérien nasal de 10 chevaux infectés par l'EHV-1 et de 11 chevaux témoins d'une ferme ayant subi une épidémie a été caractérisé à l'aide de la plate-forme Illumina MiSeq ciblant la région V4 du gène de l'ARN ribosomal 16S. Le microbiote bactérien nasal des chevaux sains et des chevaux EHV-1 était significativement différent dans l'appartenance et la structure de la communauté. Les chevaux excrétant l'EHV-1 avaient une richesse bactérienne ($P = 0,002$), une régularité ($P = 0,008$) et une diversité ($P = 0,026$) plus faibles que les chevaux en bonne santé. Les chevaux en bonne santé avaient une abondance relative plus élevée de Firmicutes et de Bacteroidetes, mais moins de Protéobactéries que les chevaux avec EHV-1 ($P < 0,05$). Cette étude fournit la base pour générer des hypothèses et des investigations sur le rôle des interactions bactériennes-virales dans la santé et les maladies des chevaux adultes.

(Traduit par Docteur Serge Messier)

Introduction

Equine herpesvirus (EHV) infection in horses can result in respiratory disease, myeloencephalopathy, abortions, and perinatal death. Infection of the upper respiratory tract (URT) with EHV results in colonization of the nasal epithelial cells (1). These processes likely involve a complex synergistic and competitive interaction between resident microbes, particularly between bacteria and viruses. Viral infections of the URT can modify the bacterial adherence and colonization and immune response of the host (2,3). Infection with EHV-1 can affect the mechanisms of defense of the URT, leading to respiratory diseases caused by opportunistic bacteria species that are part of the URT microbiota, e.g., Streptococcus equi subsp. zooepidemicus, Pasteurella spp., Actinobacillus spp., Klebsiella spp., Escherichia coli, and Bacteroides spp.

In children, virus-specific nasopharyngeal bacterial shifts have been identified during viral infections (3–5). The influence of viral colonization of the nasal epithelium on the whole bacterial composition of the URT in horses is unknown. Understanding the interaction between viruses and bacteria in the URT during infection may provide insights into the pathogenesis of respiratory infection. The structure, as well as the functional roles, of the nasal microbiota of horses undergoing viral infection should therefore be examined in more detail. The objective of this study was to profile the nasal bacterial microbiota of healthy horses and horses infected with EHV-1 from a single farm that was experiencing an EHV-1 outbreak.

Materials and methods

The farm, animals, and outbreak

During the winter of 2017, a Quarter Horse farm in southern Ontario, Canada experienced an outbreak of a neuropathogenic strain of EHV-1. Thirty adult horses and 1 adult donkey resided at the farm. Nineteen horses were kept in 1 barn, in individual stalls that allowed nose-to-nose contact, with access to outdoor paddocks during the day. Eleven horses and 1 donkey remained outside 24 h a day in 3 different outdoor paddocks, with nose-to-nose contact.

Department of Clinical Studies (Gomez, Arroyo) and Department of Pathobiology (Lillie, Weese), Ontario Veterinary College, University of Guelph, Guelph, Ontario.

Address all correspondence to Dr. Diego Gomez; telephone: +1 (226) 924-5910; e-mail: dgomezni@uoguelph.ca

This work was presented in part as a research abstract at the 2018 ACVIM Forum in Seattle, Washington, USA.

Received September 23, 2019. Accepted March 23, 2020.
Food and water sources were similar for all horses on the farm. There was no direct contact between horses that were kept in the barn and those that were housed outside at any time during the outbreak. There were 2 pregnant mares in their 9th month of gestation in the indoor group.

The farm was immediately quarantined when the first case was confirmed EHV-1 positive and all horses remaining on the premises were monitored twice a day for the presence of fever, limb edema, and nasal discharge. Fever was defined as a rectal temperature above 38.5°C.

Sample collection

Two nasal swabs were taken from all horses (healthy and those developing clinical signs compatible with EHV-1 infection) during the outbreak, 1 for diagnostic and another for microbiota analyses. A polymerase chain reaction (PCR) assay was used to screen all nasal swabs for the presence of EHV-1 genomic DNA. Nasal swabs of the horses that developed clinical signs were collected within the first 48 h of the onset of fever using a 15-mm long sterile nylon swab without transport media (FLOQSwabs; Copan Flock Technologies, Brescia, Italy). All nasal swabs were taken after wiping the nostrils with a sterile 0.9% saline solution, as described in a previous study (6). Nasal swabs were transported in a container at 4°C within 2 h of collection and stored at −80°C until analysis. Negative controls (swab only, without sample or sterile saline) were not obtained during sampling on the farm or introduced throughout the extraction, amplification, and sequencing process.

EHV-1 droplet-digital PCR assay

The DNA was extracted from nasal swabs using the OMEGA E.Z.N.A Tissue DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA) as per manufacturer’s instructions, with some modifications. The first 3 buffers were used at volumes 1.5× the amount listed in the instructions so that the fluid level completely covered the swab [300 μL of TL Buffer, 37.5 μL of OB Protease Solution, and 330 μL of BL Buffer (all from Omega Bio-Tek)]. Following the BL Buffer step, all volumes were as per the manufacturer’s instructions. The optional RNA removal step was not carried out.

Droplet digital PCR (ddPCR) was conducted by the Guelph Molecular Supercentre, Lab Services Division at the University of Guelph according to the following conditions. The ddPCR reaction mixture consisted of 1× ddPCR Supermix for Probe (Bio-Rad, Mississauga, Ontario), 96 nM each of the primers EHV1F (5′-CATGTCAACGCACCTCCA-3′), EHV1R (5′-GGGTCGGCGTTTCTGT-3′), 64 nM of EHV1 probe (5′-FAM-CCC TACGCTGCTCC-MGB-NFQ-3′) (7), and 4 μL of sample DNA in a final volume of 25 μL.

From each PCR reaction mixture, 20 μL were mixed with 70 μL of Droplet Generation Oil for Probes (Bio-Rad) in a DG8 Cartridge (Bio-Rad). The PCR droplets were then generated using a QX200 Droplet Generator (Applied Biosystems, Foster City, California, USA). From each droplet mix, 20 μL were transferred to a 96-well PCR plate. The plate was sealed with a foil heat seal using a PXI TM PCR Plate Sealer (Bio-Rad Laboratories, Hercules, California, USA). Each plate included up to 94 samples with 1 positive control (synthetic gene fragment) and 1 negative control (water). The PCR amplification was carried out on a GeneAmp PCR System 9700 at the following settings: 95°C for 10 min, followed by 48 cycles of 95°C for 20 s, and 62°C for 40 s, and 1 cycle of 98°C for 10 min.

After amplification, droplets from each well were read automatically on a QX200 Droplet Reader (Bio-Rad). The ddPCR data were acquired and analyzed with QuantaSoft software (Bio-Rad) and recorded as copies/microliter (μL). The final viral load [copies/milliliter (mL)] of each sample was determined by multiplying the average copy number per microliter (μL) of PCR mixture in each well and the sample dilution factor. A subset of samples was sent to the Animal Health Laboratory, University of Guelph, Ontario to determine the D752 variant of the DNA polymerase [open reading frame 30 (ORF30)] in the EHV-1.

DNA extraction, amplification, and sequencing of bacterial 16S rRNA gene

Total DNA was extracted from the nasal samples using a commercial kit (EZNA Stool DNA Kit, Omega Bio-Tek). The whole tip of the swab was processed through the lysis stage of extraction. The V4 region of the 16S ribosomal RNA (rRNA) gene was amplified using the forward (5′- AYTGGGYDAAAAGNG-3′) and reverse (5′- TACNVGGGTATCTAATCC-3′) primers. The primers included overhanging adapters (forward: 95′-TCGTCGGCA GCGTCAGATGTGTATAAGAGACAG-3′, reverse: 5′-GTCTCTCG GGGCTCGAGATGTGTAAGACAGACAG-3′) for annealing to Illumina universal index sequencing adapters added in a later PCR. The reaction mixture and amplification conditions were described in a previous study (8).

The PCR products were purified using magnetic beads (Agencourt AMPure XP; Beckman Coulter, Mississauga, Ontario). Illumina universal adapters (forward: 5′- AATGATACGG CGACACACC AGATCTACAC-index-TCGTCGGCAACCAGC-3′, reverse: 5′- CAA GCAGAAGAGACCGATACAGATG-index-GTCTCTCGAGAGAAGTT TCAGAGAGG-3′) were then added to the purified 16S rRNA gene product by PCR. The PCR products were assessed by electrophoresis in 1.5% agarose gel and purified with magnetic beads (Agencourt AMPure XP, Beckman Coulter). Following purification, the PCR products were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Samples were normalized to a final concentration of 2 nM. The library pool was submitted to the Genomics Facility of the University of Guelph and sequenced with an Illumina MiSeq (Illumina, San Diego, California, USA) for 250 cycles from each end. Negative controls were not included during the sampling, extraction, or sequencing processes.

Bioinformatic analysis

The Mothur v1.38 software package (Michigan State University, East Lansing, Michigan, USA) was used for sequence processing and analysis. Paired end reads were merged to fully overlapping reads and then aligned to the SILVA 16S rRNA reference database (9). Sequences inconsistent with the target amplicon size (240 bp) or containing ambiguous base calls or those with runs of homopolymers > 8 base pairs were removed. Sequences misaligned with the V4 region were also removed. Chimeras were identified with UCHIME and then removed, as were sequences belonging to nonbacterial domains. The remaining sequences were assigned to
operational taxonomic units (OTUs) using an open OTU-picking approach, with a distance limit of 97% similarity. The Ribosomal Database Project Classifier was used for taxonomy assignment (Michigan State University).

**Statistical analysis**

Subsampling was completed to normalize sequence number by random selection of a number of sequences that corresponded to the lowest number of reads for any sample. The relative abundances of the main phyla, classes, orders, and families (median relative abundance > 0.1%) and the main genera (median relative abundance > 0.05%) were then calculated and compared using the non-parametric Mann-Whitney U-test. P-values were adjusted for multiple comparisons with Benjamini-Hochberg False Discovery Rate using statistical software (R Core Team, 2013, R Foundation for Statistical Computing, Vienna, Austria) to generate q-values. A q of < 0.05 was considered statistically significant.

Good’s coverage value was used to assess sampling coverage (10). Richness, evenness, and diversity indexes were calculated using the Chao1, Shannon’s evenness, and inverse Simpson’s indexes, respectively. Comparisons among groups were made using Wilcoxon rank-sum test. The core microbiota was investigated by identifying genera with relative abundances of at least 1% in all samples from a group.

Similarities of the bacterial microbiota membership and structure were calculated using a metric based on OTU richness (Jaccard index) (11) or abundance (Yue and Clayton index) (12), respectively. Differences in community membership and structure among groups were assessed using the Unweighted-Unique Fraction Metric (Unweighted-UNIFRAC) analysis, parsimony test, the analysis of molecular variance (AMOVA), and the homogeneity of molecular variance analysis (HOMOVA). Dendrograms were created to visualize similarities among groups (FigTree v1.4.0.1. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, Scotland). Clustering of the groups was represented by principal coordinate analysis plotted using statistical software (JMP 12, SAS Institute, Cary, North Carolina, USA).

Linear discriminant analysis effect size (LEfSe) (13) was used to identify bacterial taxa that were enriched in nasal samples from control and EHV-1 horses, based on \( P < 0.05 \) and linear discriminant analysis (LDA) score of > 3.0. Dirichlet multinomial mixture model (DMM) was used to assess the number of different meta-communities into which the data could be clustered (14). The number of different meta-communities was determined by selecting the number of components that gave the minimum Laplace approximation to the negative log model. Samples were assigned to their community type based on the maximum posterior probability.

**Results**

**The horses and the outbreak**

During the outbreak, 18/19 horses kept in the barn developed clinical signs, including fever (14/18), limb edema (10/18), and nasal discharge (1/18). Two pregnant mares aborted during the outbreak. As 10/18 horses that developed clinical signs tested positive for EHV-1 in the ddPCR of nasal samples at the time of sampling, only 10 EHV-1 positive horses were enrolled in this study.

The anamnesis of the 10 EHV-1 positive horses was similar: clinical signs started with fever (\( n = 10 \)) and then the horses developed limb edema (\( n = 6 \)). Both mares that aborted did so after 2 d of fever, which generally lasted for 3 to 5 d, but was controlled after NSAIDs were administered. None of the 10 EHV-1 positive horses developed neurological signs. All samples positive for EHV-1 on ddPCR were also positive on quantitative PCR (qPCR) at the Animal Health Laboratory, University of Guelph. In addition, all horses with EHV-1 carried the D752 variant of the DNA polymerase (ORF30). None of the 11 healthy control horses had detectable levels of EHV-1 DNA in nasal samples.

**Analysis of 16S rRNA gene sequencing**

A total of 858 263 reads were obtained with a mean of 40 860 reads per horse (standard deviation: 21 132, median: 38 263, and range: 11 977 to 107 141). A random subsample of 11 977 reads per sample was used to normalize data. Subsampling was considered adequate, as evidenced by the coverage of 99.9% obtained for all samples.

**Alpha diversity**

Horses infected with EHV-1 had significantly lower bacterial richness, evenness, and diversity than control horses (Figure 1).
Relative abundance

A total of 33 different phyla were identified, but Proteobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, Spirochaetes, Fibrobacteres, and Bacteroidetes accounted for 90% of sequences (Figure 2). These phyla were identified in control and EHV-1 horses at >1% of the total number of sequences. Comparison between control and EHV-1 horses identified a higher relative abundance of Firmicutes ($P = 0.04$), Bacteroidetes ($P = 0.01$), and Fusobacteria ($P = 0.01$) in control horses. However, high inter-individual variation was identified within each group (Figure 2A).

While 25 different classes, 43 orders, and 135 families were identified, only 11, 27, and 50 accounted for $\approx 0.1\%$ of sequences overall, respectively. The relative abundance of bacterial taxa that were significantly different between control and EHV-1 horses is presented in Table I. The relative abundance of the most abundant bacterial genera identified in control and EHV-1 horses is presented in Table II.

Overall, 850 genera were detected. A total of 126 of those were present at a relative abundance of $>0.05\%$. The relative abundance of the main bacterial genera found in control and EHV-1 horses is presented in Figure 2B, which shows the high inter-individual variation that was identified within each group.

LEfSe analysis: Enriched phylotypes

When comparing control and EHV-1 horses, 19 bacterial taxa enriched in control horses and 5 enriched in EHV-1 horses were identified. Enriched phylotypes in control horses were predominantly from the phylum Firmicutes, whereas most from EHV-1 horses were Proteobacteria and Actinobacteria (Figure 3). The genera Brachybacterium, Dietzia, Arthrobacter, Psychrobacter, and Moraxella were significantly associated with EHV-1 infection, whereas...
Lactobacillus, and unclassified genera of the family Clostridiales and Lachnospiraceae among others were enriched in control horses (Figure 3).

**Bacterial community analysis**

Nasal bacterial microbiota of healthy horses and horses with EHV-1 were significantly different in community membership (Jaccard index) and structure (Yue and Clayton index) (Parsimony and AMOVA; *P* < 0.05). These differences in community membership and structure were visualized by principal coordinates analysis (PCoA) plots (Figure 4). Nasal bacterial microbiota of healthy horses from different outdoor paddocks was similar in community membership (Jaccard index) and structure (Yue and Clayton index) (Parsimony and AMOVA; *P* > 0.05) (data not presented).

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Control</th>
<th>EHV-1</th>
<th>q-value</th>
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<tbody>
<tr>
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<td></td>
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<tr>
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<td>0.07 (0 to 0.2)</td>
<td>0.019</td>
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<td>Firmicutes</td>
<td>36 (20 to 53)</td>
<td>20 (2.8 to 50)</td>
<td>0.047</td>
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<td>Class</td>
<td></td>
<td></td>
<td></td>
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<td>0.017</td>
</tr>
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<td>Sphingobacteriales</td>
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<td>0.2 (0.03 to 1.6)</td>
<td>0.017</td>
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<td>Aeromonadales</td>
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<td>0.06 (0 to 0.3)</td>
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<tr>
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<td>0.1 (0.03 to 0.77)</td>
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<td>0.08 (0 to 0.2)</td>
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</tr>
<tr>
<td>Succinivibrionaceae</td>
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<td>Alteromonadaeace</td>
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<tr>
<td>Flavobacteriaceae</td>
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<td>0.04</td>
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<td>Enterococcaceae</td>
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<tr>
<td>Viridibacillus</td>
<td>0.2 (0.1 to 1.2)</td>
<td>0.05 (0.02 to 0.5)</td>
<td>0.032</td>
</tr>
<tr>
<td>Clostridiaceae_1_uncl.</td>
<td>0.3 (0.1 to 0.7)</td>
<td>0.1 (0 to 0.3)</td>
<td>0.032</td>
</tr>
<tr>
<td>Selenomonas</td>
<td>0.1 (0.03 to 0.7)</td>
<td>0.04 (0 to 0.1)</td>
<td>0.032</td>
</tr>
<tr>
<td>Roseburia</td>
<td>0.1 (0.02 to 0.3)</td>
<td>0.04 (0 to 0.1)</td>
<td>0.032</td>
</tr>
<tr>
<td>Alkaliphilus</td>
<td>0.1 (0.02 to 0.2)</td>
<td>0.01 (0 to 0.1)</td>
<td>0.036</td>
</tr>
<tr>
<td>Peptostreptococcus</td>
<td>0.1 (0.01 to 0.5)</td>
<td>0.05 (0 to 0.3)</td>
<td>0.044</td>
</tr>
<tr>
<td>Lysinibacillus</td>
<td>0.05 (0.02 to 0.6)</td>
<td>0.02 (0 to 0.7)</td>
<td>0.044</td>
</tr>
<tr>
<td>Desulfovibrio</td>
<td>0.03 (0 to 0.02)</td>
<td>0 (0 to 0.02)</td>
<td>0.044</td>
</tr>
<tr>
<td>Succinimesticium</td>
<td>0.05 (0 to 0.4)</td>
<td>0.01 (0 to 0.09)</td>
<td>0.044</td>
</tr>
</tbody>
</table>

*Only significantly different taxa are presented.

q-value — *P*-value adjusted based on the Benjamini-Hochberg False Discovery Rate; uncl — an unclassified taxonomy within the respective taxonomic group.
It was found that the nasal bacterial microbiota of healthy horses is richer and more diverse than that previously reported using culture-based methodology (15,16). Proteobacteria, Firmicutes, and Actinobacteria phyla dominated the nasal microbiota of healthy horses, similar to what had been reported in humans (17), pigs (18), dogs (19), and cattle (20). The same taxa were identified as the most abundant phyla in nasopharyngeal samples of a small group of healthy horses (n = 6) in a previous study (6).

The most abundant genera in the nasal cavity of healthy horses in this study were Nicoletella, Treponema, Streptococcus, Staphylococcus, Lactobacillus, and genera from the order Clostridiales and the families Ruminococcaceae, Lachnospiraceae, and Clostridiales. Nicoletella spp. have been isolated from the respiratory tract of healthy horses of different ages and breeds and those with signs of bronchopneumonia and chronic airway inflammation (21–23). Moraxella has been associated with pulmonary disease in combination with other bacterium, e.g. Streptococcus equi var. zooepidemicus (22,23).

Our study failed to detect differences in the relative abundance of Nicoletella in healthy and EHV-1 infected horses. Similar to our study, Streptococcus and Staphylococcus genera have been identified as major components of the nasopharynx microbiota of healthy horses (6,16), while Lactobacillus and genera from the order Clostridiales have been detected in nasal samples of healthy pigs (18).

### Table II. Relative abundance (median in percentage and ranges) of the 10 more abundant bacterial genera identified in nasal samples of healthy horses and horses infected with equine herpesvirus 1 (EHV-1).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Healthy</th>
<th>EHV-1</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicoletella</td>
<td>2.4 (0 to 51)</td>
<td>41 (0.6 to 93)</td>
<td>0.09</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>0.8 (0.1 to 42)</td>
<td>1 (0.3 to 58)</td>
<td>0.34</td>
</tr>
<tr>
<td>Unclassified Clostridiales</td>
<td>4.4 (0.6 to 9)</td>
<td>1.3 (0.2 to 9)</td>
<td>0.07</td>
</tr>
<tr>
<td>5 genus incertae sedis</td>
<td>5 (0.2 to 7)</td>
<td>1.5 (0.1 to 11)</td>
<td>0.2</td>
</tr>
<tr>
<td>Moraxella</td>
<td>0.1 (0 to 13)</td>
<td>2.7 (0 to 20)</td>
<td>0.09</td>
</tr>
<tr>
<td>Unclassified Lachnospiraceae</td>
<td>3.1 (0.5 to 8)</td>
<td>0.7 (0.1 to 5)</td>
<td>0.05</td>
</tr>
<tr>
<td>Unclassified Ruminococcaceae</td>
<td>3.2 (0.5 to 7)</td>
<td>1 (0.1 to 4)</td>
<td>0.07</td>
</tr>
<tr>
<td>Treponema</td>
<td>2.3 (0.2 to 6)</td>
<td>0.6 (0 to 3.6)</td>
<td>0.09</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>2.3 (0.2 to 6)</td>
<td>0.5 (0.07 to 2.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>0.3 (0.1 to 10)</td>
<td>0.8 (0.1 to 14)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

q-value — P-value adjusted based on the Benjamini-Hochberg False Discovery Rate.
Limitations of the results reported here include the lack of inclusion of negative controls during the microbiota assessment and the descriptive nature of the study with sampling at a single time point. The lack of inclusion of negative controls during the sample collection and processing limited the interpretation of our results. Several studies have reported the presence of contaminant DNA during microbiota analysis of low biomass samples and demonstrated how this can skew the results and confound their interpretation (24–26).

Currently, there are no systematic recommendations or requirements for examining or reporting contaminants within microbiota studies despite the information collected from different investigations describing amplification of contaminants in microbiota studies (26). Contaminant DNA can originate from the sampling, extraction, or sequencing processes. In order to monitor the background levels of contaminant DNA, the following controls should have been included: sampling blank controls, such as blank swabs and swabs exposed to the sampling environment; DNA extraction blank controls to monitor for contaminant DNA present in the extraction kits and reagents; and no template amplification controls to monitor for DNA contaminant originated from reagents and laboratory environment during library preparation and sequencing (26). The use of negative controls would have provided a semi-quantitative assessment of DNA contaminants to identify taxa that can be removed from the analysis or compared to nasal samples to determine their impact on the results reported here (26).

The descriptive nature of the study prevented us from extrapolating our results to the overall population as our results originate from a group of horses maintained in specific conditions at one particular time. The single time point sampling precluded the analysis of temporal changes in the bacterial communities of horses infected with EHV-1. A longitudinal characterization of the nasal microbiota of EHV-1 horses would have been ideal to determine whether those changes were transient or permanent.

Despite some statistically significant differences identified in this study, there was large variation between horses, which made comparisons between groups difficult. For instance, Proteobacteria was the most abundant phylum in some healthy horses, while Firmicutes or Actinobacteria predominated in others. Similarly, large individual variation was observed with regard to the most abundant genera. For example, while 5 of 11 healthy horses had a relative abundance of *Nicoletella* (> 25%), 6/11 had lower than 5% and, when the data were summarized, healthy horses had a low relative abundance of *Nicoletella* (< 5%) (Figure 2B).

This high biological inter-subject variability, especially in healthy subjects, can be a major confounding factor in microbiota studies (4,27). Our data highlight the fact that summaries of bacterial community, as seen in Figures 2A and 2B, must be interpreted cautiously, as they can hide the high degree of individual variability within each group and do not necessarily represent actual patterns seen at the individual level. Therefore, our analyses highlight the need to examine beyond simple comparisons of relative abundances when trying to interpret the microbiota because grouped summaries of relative abundance can result in spurious associations, especially when the sample is small.

There are no explanations for the high inter-individual variability, but differences in age, breed, diet, source of the diet components, pregnancy status, and exercise are associated with changes in the gut microbiota of different species. All horses involved in this outbreak were adults (> 3 y), Quarter Horses (except for 1 donkey), fed the same hay, and did not have access to pasture. The level of exercise was different among the horses reported here, which could potentially explain the variability among individuals. The nature of the...
results presented, however, prevents us from drawing further conclusions about the reasons for the high inter-individual variability.

The nasal bacterial microbiota of healthy horses and EHV-1 infected horses in the outbreak reported here differed significantly in community membership and structure. The differences detected between groups could have resulted from the viral infection, although housing was different between groups involved in this outbreak, and the environmental variable could therefore have confounded our results (20,28). Interestingly, the changes in alpha (lower richness and diversity) and beta diversity observed in the EHV-1 positive group were similar to those identified in humans after viral infection (4,5,27,29) and cattle with respiratory disease (30). While these findings suggest that the observed differences could have occurred as a consequence of EHV-1 infection, the environmental differences among horses should be considered when interpreting the results reported here (20,28).

The planning of how this outbreak investigation was conducted prevented us from drawing conclusions about temporal changes, but studies in humans have demonstrated that changes occur in bacterial composition of the nasal microbiota during the symptomatic rather than the asymptomatic phase of viral infection with human rhinovirus (5). This finding supports the hypothesis that the community structure of the nasal bacterial microbiota changes during viral infection, which is possibly explained by the overgrowth of potential pathogens triggered by the viral infection (5,27,28).

In horses, the most well-known interaction leading to respiratory disease is the synergism between influenza virus or EHV-4 and Streptococcus equi var. zooepidemicus (31). The postulated mechanisms explaining how viral infections can predispose to bacterial colonization include decreased mucociliary clearance, loss of barrier function, upregulation of adhesion proteins, altered expression of antimicrobial peptides, and impairment of neutrophil and monocyte function (32). It can be hypothesized that infection of the upper respiratory tract with EHV-1 in the horses reported here had an impact on the respiratory epithelium and immune system, which led to a shift in the bacterial communities present in the nasal cavity of affected horses.

Linear discriminant analysis effect size (LDFSe) identified enrichment of Moraxella, Dietzia, Psychrobacter, Brachibacterium, and Arthrobacter in EHV-1 infected horses. Arthrobacter, and Brachibacterium are genera of bacteria that are commonly found in soil, feces, and a wide range of moist cold habitats. Its presence appears to be associated with environmental contamination of the nasal cavity (33). However, the role of this likely transient bacteria microbiota in health remains to be determined. Psychrobacter and Moraxella are genera of the family Moraxellaceae that has been detected in the upper respiratory tract of healthy cattle (20) and horses (6). Although our analysis showed both Psychrobacter and Moraxella as different organisms, it is possible that both bacteria are in fact the same organism, as the low resolution of the next generation of sequencing makes it difficult to detect differences at the genera and species level. Similarly, Moraxella can be detected in the nasal and nasopharyngeal samples of healthy children (5), dogs (19), cats (34), and cattle (20). It has also been associated, however, with increased risk of developing asthma and pneumonia in children (35,36) and respiratory disease in cattle (37).

A higher abundance of Moraxellaceae family members was found in humans after infection with human rhinovirus (5), acute respiratory infection (17), and viral colonization (38). A higher abundance of Moraxellaceae was also identified in cats with upper respiratory tract diseases (34). These findings support the hypothesis of persistent bacterial outgrowth, especially genera from the Moraxellaceae family, after viral infection (5,38). No studies have associated the presence of Moraxella spp. in horses with respiratory disease, but this bacterium can be isolated from horses with lymphoid hyperplasia (grades III and IV) (16) and in horses with conjunctivitis (39). Given the importance of this genus as a causative agent for disease in other species and in different organ systems in horses, further investigation is warranted in order to clarify the role of Moraxella during viral infection of the respiratory system of horses.

The genus Dietzia was also enriched in nasal samples of horses infected with EHV-1. The Gram morphology and colony appearance of the species of this genus is remarkably similar to Rhodococcus equi (40). While the pathogenic role of this bacterium has not been completely clarified, a recent report in human medicine suggested this genus as a possible opportunistic bacterium (40). Further studies are necessary to establish the role of these taxa in the homeostasis of the respiratory system of horses.

In conclusion, it was observed in this study that the nasal cavity of healthy horses is inhabited by a large variety of bacteria with significant individual variation. Acute infection with EHV-1 appears to affect the nasal microbiota, as changes were seen in the diversity, membership, and structure of the bacterial communities. However, given the limitations of this study, e.g., environmental differences between groups, experimental studies controlling for environmental conditions are needed to confirm our results.

Acknowledgment

The authors thank Joyce Rousseau for her input and advice during the process of carrying out the experiments described in this article.

References

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Description of the bacterial microbiota of anal sacs in healthy dogs
Camylene C Bergeron, Marcio C. Costa, Lucilene B. de Souza, Frédéric Sauvé

Abstract
The aim of the present study was to characterize the bacterial microbiota of anal sacs in healthy dogs using NGS. Swabs were used to sample the rectum and secretions from each anal sac in 15 healthy dogs. DNA was extracted from swabs and the V4 hypervariable region of the 16S rRNA gene was amplified and sequenced with Illumina MiSeq. Overall, 14 different bacterial phyla were identified in the rectum and in both anal sacs, the 5 main ones being Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. The rectum had higher microbial diversity and richness than the left and right anal sacs. Community membership and structure significantly differed between the rectum and both anal sacs, but not between the right and the left anal sacs. This study showed that the diversity and richness of the bacterial microbiota of the anal sacs in dogs is greater than what has been reported in previous studies with culture-based methods. In conclusion, the bacterial microbiota of the anal sacs in dogs varies between individuals and differs from the rectal bacterial microbiota.

Résumé
L’objectif de la présente étude était de caractériser le microbiote bactérien des sacs anaux de chiens en santé en utilisant le séquençage d’ADN à haut débit. Des écouvillons ont été utilisés pour échantillonner le rectum et les sécrétions provenant de chaque sac anal chez 15 chiens en santé. L’ADN a été extrait des écouvillons et la région hypervariable V4 du gène codant pour l’ARN ribosomique 16S a été amplifiée et séquencée avec Illumina MiSeq. En tout, 14 phyla bactériens différents ont été identifiés dans les sacs anaux droit et gauche et le rectum, les cinq principaux étant Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria et Fusobacteria. Le microbiote bactérien du rectum avait une diversité et une richesse plus importantes que le microbiote bactérien des deux sacs anaux. L’appartenance à la communauté et sa structure différaient significativement entre le rectum et les sacs anaux, mais pas entre le sac anal droit et le sac anal gauche. Cette étude a démontré que la diversité et la richesse du microbiote bactérien des sacs anaux chez le chien sont plus importantes que ce qui a été rapportées dans les études précédentes avec des cultures. En conclusion, le microbiote bactérien des sacs anaux chez le chien varie d’un individu à l’autre et diffère de celui du rectum.

Introduction
Dogs have a pair of anal sacs, which are skin invaginations between the muscles of the internal and external anal sphincters (1,2). They are connected to the anocutaneous junction by a duct where the secretions produced by the apocrine and sebaceous glands in the anal glands are evacuated during defecation. The full content of the anal sacs’ secretions is a mixture of glandular secretions, desquamated keratinocytes from the stratified squamous epithelium lining the anal sacs and ducts, cellular debris, and resident microorganisms such as bacteria and yeasts (1–4). Some cytology studies also reported the presence of leucocytes and erythrocytes in normal anal sacs (2,4,5). The function of anal sacs is not clearly established, but it has been suggested that the volatile compounds of anal sac secretions may have a role in scent communication in dogs (2,6).

Dogs can be affected by diseases related to anal sacs, such as impaction, inflammation, infection, or neoplasia. Some of these conditions may require regular anal sac expression, local or systemic antibiotic treatment, dietary modification, or in more severe or refractory cases, surgery (5,7). Unfortunately, the pathophysiology and risk factors related to anal sac diseases are mostly uncertain, making the prevention of these conditions more difficult (8).

Bacterial microbiota is defined as the bacteria living in a specific environment (9). It is very important for maintenance of health: it participates in digestion and detoxification, competes with pathogens to prevent their colonization, and interacts with the immune system (10–13). Interactions between bacterial microbiota and the immune system are essential in order to have adequate innate and adaptive immune responses (13–15). Moreover, the microbiota stimulates the immune system at the surface of the skin and the digestive, respiratory, and genitourinary epithelia and mucosa to help protect these areas against pathogens (16). Thus, imbalances in bacterial microbiota have been associated with the pathogenesis of certain diseases (16–18).

In order to determine the role of bacterial microorganisms in healthy and diseased anal sacs, we must first better define the bacterial microbiota in anal sacs of healthy dogs. Previous studies using culture-based approaches have identified staphylococci,
streptococci, micrococci, *Bacillus* spp., *Escherichia coli*, *Proteus* spp., and *Pseudomonas* spp. as normal commensal bacteria in anal sacs, but to the best of the authors’ knowledge, no studies have been performed on the content of anal sacs in dogs using new methods of DNA sequencing (2,19,20).

The objectives of this study were to describe the anal sac microbiota of healthy dogs using next-generation DNA sequencing (NGS) and to investigate the influence of rectal bacteria in the composition of the anal sac microbiota.

**Materials and methods**

**Animal selection**

This study was approved by the Faculty of Veterinary Medicine of the University of Montreal’s Animal Care Committee. The MIRA Foundation, a non-profit organization that offers free guide dogs and service dogs, signed a written consent form to participate in this study.

The breeding dogs lived with foster families. Several days before giving birth, the pregnant bitches were transferred to the MIRA Foundation’s facility (MIRA Campus) where they gave birth. The puppies stayed in this facility for 9 wk. At 9 wk of age, the puppies were brought to a foster family with whom they stayed until they reached 12 to 18 mo of age. Then, the dogs came back to MIRA Campus to be trained for assistance (visual and mobility impairment, autism spectrum).

Fifteen healthy dogs from the MIRA Foundation were enrolled in this study. Because the MIRA Foundation breeds and manages their own dog colony, the dogs had a related genetic background. Thirteen Laberneses and 2 Labrador retrievers aged 1 to 11.8 y (median = 1.3 y) were enrolled in this study and fed similar diets (Table I). Dogs 2 to 6, 8, and 14 were all in contact, living together at MIRA Campus. Dogs 10, 12, and 13 were in lactation and lived in the same facility but in different pens at MIRA Campus. The remaining dogs (1, 7, 9, 11, and 15) lived in separate houses.

To be included in the study, the dogs could not be bathed and could not receive systemic or topical drugs (antibiotics, anti-inflammatories, essential fatty acids) 1 and 3 mo before the study, respectively. The dogs had to be healthy based on physical examination (no clinical signs or physical examination findings consistent with cutaneous or systemic disease, or with neoplasm).

**Sample collection**

Three swabs were collected on the same day on each dog: one from the rectum and one from each anal sac (left and right). All the samples were collected from November 2018 to March 2019.

The perianal area was cleaned with sterile gauzes and 4% chlorhexidine (Dermachlor 4; Dechra, Pointe-Claire, Quebec). Two minutes after cleaning the area, a sample from the rectum was obtained by inserting a sterile flocked swab (FLOQSwabs; Murrieta, California, USA) 2 cm into the rectum.

After putting on sterile gloves, sterile lubricant was applied to the index finger, which was gently inserted into the anus. With the thumb outside the dog’s anus, the left anal sac was expressed by bringing the index and thumb together so that the content of the anal sac got through the duct opening on the left side of the anus. The first drops were not collected, to decrease the risk of contamination and to be more representative of the content of the anal sac. The secretions were collected with a sterile flocked swab. The secretions of the right anal sac were collected in the same fashion. Before collecting the secretions from the right anal sac, the perianal area was cleaned again with 4% chlorhexidine and the sterile gloves were changed. All the samples were transported, refrigerated, and subsequently frozen at −80°C until DNA extraction.

**DNA extraction and sequencing**

The DNA extraction was performed on all samples, including 2 unused swabs (negative controls), with a commercial kit DNeasy PowerSoil (Qiagen, Hilden, Germany) as recommended by the manufacturer (21). In the first step of DNA extraction, the tip of the swab was cut off and deposited in the provided tube containing the beads. A solution provided by the commercial kit was added to allow cell lysis and the tube was vortexed for 10 min. The supernatant was then transferred to another tube and the remaining steps were followed as recommended by the manufacturer. Previously reported primers (515 forward paired with 806 reverse) were used to amplify the V4 hypervariable region of the bacterial 16S ribosomal RNA gene by polymerase chain reaction (PCR) (22). Sequencing was performed at the Genome Quebec Innovation Centre. The Illumina MiSeq IEMFile version 4 platform was used for sequencing using the V2 reagent kit (2 × 250 cycles). Sequences are available at the NCBI Sequence Read Archive under accession number PRJNA681230.

The software mothur was used to perform the bioinformatic analysis (23). It clustered the good quality reads in operation taxonomic units (OTUs) at the genus level (> 94% similarity). The classification of OTUs was performed using the Ribosomal Database Project databank.

**Statistical analysis**

The alpha diversity was evaluated with the Chao index (estimator of richness), Shannon index, and inverse Simpson index (diversity indices) (9,24). The Anderson-Darling test was performed to assess the normality of data distribution and results from the inverse Simpson index were transformed into log to obtain a normal distribution. A 2-way analysis of variance was performed with the sampling site and the dog as the independent variables, followed by a Tukey test to identify significant differences in the alpha diversity indices, with $P < 0.05$ considered significant.

Beta diversity, which is the comparison of similarities between samples, was assessed using the Jaccard index, which evaluates community membership (i.e., which bacteria are present or absent in a community), and by the Yue and Clayton index, which evaluates community structure (i.e., which bacteria are present based on their abundance in a community) (9,25). The analysis of molecular variance was used to compare the community membership and community structure between groups. A $P < 0.05$ was considered significant. Principal coordinate analysis (PCoA) plots were obtained to visualize the similarities between groups (rectum and left and right anal sacs) (9). Linear discriminant analysis effect size (LEfSe) was used to find significant associations between relative abundances across anatomical sites (26).
**Results**

Ten and 9 bacterial phyla were identified in the left and right anal sacs, respectively, while 12 bacterial phyla were identified in the rectum. The predominant phyla in the left and right anal sacs and rectum were Firmicutes (60.4%, 60.1%, and 26.2%, respectively), Bacteroidetes (20.2%, 26.0%, and 21.3%, respectively), Proteobacteria (17.4%, 10.2%, and 20.5% respectively), Fusobacteria (1.1%, 3.3%, and 4.1%, respectively), and Actinobacteria (0.9%, 0.4%, and 27.7%, respectively) (Figure 1). The predominant genera found in the left and right anal sacs were *Enterococcus* (34.3% and 31.5%, respectively), *Bacteroides* (16.5% and 20.6%, respectively), and *Proteus* (10.9% and 8.5%, respectively), while in the rectum, the predominant genera identified were *Corynebacterium* (21.5%), *Prevotella* (13.8%), and *Lactobacillus* (8.5%) (Figure 2). No detectable bacterial DNA was amplified from the blank samples after PCR. The *Corynebacteriaceae* family was significantly associated with rectal samples, but no other discriminant markers were found with the LefSe analysis.

The richness (number of bacteria genera observed) was significantly lower in the left and right anal sacs compared to the rectum ($P < 0.001$ and $P < 0.001$, respectively), but there was no statistical difference between the right and left anal sacs ($P = 0.161$) (Figure 3). The Chao index, which is an estimator of true richness, was significantly lower in the left and right anal sacs compared to the rectum ($P < 0.001$ and $P < 0.001$, respectively), but there was no significant difference between both anal sacs ($P = 0.337$). The diversity was significantly lower in the left and right anal sacs compared to the rectum according to the Shannon index ($P < 0.001$ and $P < 0.001$, respectively) and inverse Simpson index ($P = 0.002$ and $P = 0.001$, respectively). There was no statistical difference in the diversity between the right and left anal sacs according to the Shannon index ($P = 0.950$) and inverse Simpson index ($P = 0.981$).

The PCoA plots revealed clear differences between anal sacs and rectum community structure, and to a lesser extent, between anal sacs and rectum community membership (Figure 4a). When considering individual factors such as diet, place of housing, and lactation, no clustering was observed. Interestingly, community structure of the left and right anal sacs clustered according to the dog in some of the samples, indicating a possible influence of environment in those dogs (Figure 4b). The statistical comparison of community beta diversity showed a significant difference in community membership of the rectum compared to the left and right anal sacs ($P = 0.032$ and $P < 0.001$, respectively), but not between both anal sacs ($P = 0.972$). There was also a statistical difference in community structure of the rectum compared to the left and right anal sacs ($P < 0.001$ and $P < 0.001$, respectively), but there was no statistical difference between both anal sacs ($P = 0.660$).

**Discussion**

This study shows that the bacterial microbiota of anal sacs is much more diverse and richer than previously reported in studies using culture-based methods (2,19,20). The bacteria mainly identified in the healthy anal sacs with standard culture media were *Micrococcus* spp., *Staphylococcus* spp., *Streptococcus faecalis*, and *Escherichia coli* (2,19,20). *Proteus mirabilis*, beta-hemolytic *Streptococcus* spp., *Bacillus* spp., and *Pseudomonas aeruginosa* have also been reported (2). Although a major limitation of NGS is the low resolution at lower taxonomic levels (i.e., species level) (27), DNA consistent with all those bacterial genera, except *Bacillus* spp., could be detected in the anal sacs of the dogs in the present study. While *Micrococcus* spp. have been repeatedly isolated from the anal sacs of healthy dogs with culture-based methods, this genus was only detected in left anal sacs at very low relative abundances (~0.00012%) in the present study (2,19). This finding could be explained by the fact that the sequenced region used in the present study may not be ideal for selecting *Micrococcus* spp. (primer bias). The primer bias could also explain why *Bacillus* spp. was not found in the anal sacs in this study. The low number of cases in this study could also be an explanation. In addition, many other bacteria were detected by NGS, likely because these bacteria do not grow or grow poorly on standard culture media.
Considering that anal sacs in dogs are skin invaginations, it could have been hypothesized that bacterial communities in anal sacs and in skin microbiota are similar (1,2). In some studies reporting on skin microbiota in healthy dogs, samples were taken from the perianal area, which is the closest region to the anal sacs (28–30). The predominant bacterial phyla reported in the perineal region were similar to the ones found in the anal sacs of dogs in the present study (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria). The main bacteria at the genus level found in the left and right anal sacs were *Enterococcus* (mean relative abundance of 34.3% and 31.5%, respectively), while the mean relative abundance of this genus in the perianal area was less than 2% in skin microbiota studies (28–30). This difference observed at the genus level is likely caused by different environmental conditions between the anatomical sites.

Previous studies have investigated the microbiota of the rectum or feces in dogs (31–35). Overall, the main bacterial phyla identified from feces samples taken from the rectum at necropsy or following dog defecation were the same as the ones found in this study, namely, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria (31–35). However, the relative abundance of phyla varied across studies. The relative abundance of Actinobacteria phylum is the one that contrasted most strongly with the results of the present study. In previous studies, the mean or median relative abundance of Actinobacteria was less than 2%, whereas in the present study, it was 27.7% in dogs; this may be due to the materials and methods used in each study, such as the DNA extraction kit or the primer sets (31–35).

The present study also aimed to determine whether the microbiota of the rectum would correlate with the microbiota of the anal sacs, since these structures are very close to each other. Indeed, anal sacs in dogs are located laterally to the anus and communicate with the mucocutaneous junction of the anus via a duct (1,2). However, the present study showed that the microbiota of healthy anal sacs and rectum are different. It has been shown that there is a significant difference between the bacterial composition of the mucous membranes and the haired skin regions (30). Since the rectum is a mucous membrane and the anal sacs are an invagination of the skin, this could be a factor in explaining the difference between the bacterial microbiota of the rectum and the anal sacs (2,33). However, there are probably other more important factors that could explain this difference, such as secretions from the sebaceous and apocrine glands in the anal sacs, the presence of stool in the rectum, and environmental discrepancies (e.g., temperature, humidity, oxygen availability, etc.) (2,3). The *Corynebacteriaceae* family was overrepresented in the rectal samples according to the LEfSe. The lack of other statistically significant differences is probably a consequence of the interindividual variability on community structure (and relative abundances) and the low sample size used in the study.

The selection of a homogeneous population of dogs for this study was aimed to reduce confounding factors that could potentially bias results. Yet, a high interindividual variability was demonstrated. It
has been shown that breed may affect the skin microbiota of dogs; therefore, further studies enrolling more diverse populations (i.e., different breeds, ages, and environments) are needed before the results of this study can be extrapolated to all dogs (29).

Some studies have shown that diet can influence fecal microbiota of dogs (34,35). The animals used in this study were fed similar diets. Although the study was not specifically designed to assess the impact of environmental factors on the microbiota of anal sacs, the PCoA plots suggest that the bacterial composition of these animals was not influenced by location, diet, or lactation. The design of the study and the small number of dogs enrolled preclude further extrapolations of results.

In conclusion, compared to previous studies using culture-based methods, a larger number of bacterial genera and a more diverse bacterial microbiota were present in the anal sacs of healthy dogs using NGS. The bacterial communities were similar between the left and right anal sacs, but significantly different between the anal sacs and the rectum. In order to better understand the role of the bacteria in anal sac diseases, future studies comparing the microbiota of healthy and diseased anal sacs are required.

Acknowledgments

This study was financially supported by the Companion Animals Health Fund of the Faculty of Veterinary Medicine of the University of Montreal, Zoetis, and the Fonds du Centenaire of the Faculty of Veterinary Medicine of the Université de Montréal. We would like to thank the MIRA Foundation for their assistance in recruiting cases and for allowing their dogs to participate to this study.

References


Figure 4. Principal coordinate analysis plots of the community structure between sampling sites and dogs. A — Comparison of community structure of rectum and anal sacs. B — Community structure of both anal sacs in each dog (connected by lines mainly when they were not clustering).
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Incidence of Shiga toxin-producing *Escherichia coli* in diarrheic calves and its susceptibility profile to antimicrobials and *Eugenia uniflora* L.

Marcelo F.G. Boriollo, Bianca S. Moreira, Mateus C. Oliveira, Taiane O. Santos, Luciana R.A. Rufino, Nelma M.S. Oliveira

**Abstract**

The aim of this study was to evaluate the occurrence of Shiga toxin (*stx*)-producing *Escherichia coli* (STEC) in diarrheic newborn calves, as well as the resistance profile of this microorganism against antimicrobials routinely used in veterinary therapy. The antimicrobial profile of *Eugenia uniflora* against *E. coli* clinical isolates was also analyzed. Specimens from the recto-anal junction mucosa were investigated by using chromogenic medium and identification of *E. coli* was done using microbiological methods (Gram staining, indole test, methyl red test, Voges-Proskauer test, citrate test, urease test, and hydrogen sulfide test). The *stx1* and *stx2* genes corresponding to the STEC pathotype were evaluated by using polymerase chain reaction and electrophoresis. The susceptibility profile to antimicrobial agents commonly used in veterinary therapeutic practice and the antimicrobial effect of lyophilized hydroalcoholic extract of *E. uniflora* L. leaves against *E. coli* clinical isolates were evaluated by disk diffusion and microdilution methods. Shiga toxin-positive *E. coli* was identified in 45% of diarrheic newborn calves (*stx1* = 23.2%, *stx2* = 4.0%, *stx1* + *stx2* = 18.2%). The frequency of *stx*-positive *E. coli* in the bacterial population was equal to 17.0% (168/990 clinical isolates): 97 (9.8%) *stx1*-positive *E. coli*, 12 (1.2%) *stx2*-positive *E. coli*, and 59 (6.0%) *stx1* + *stx2*-positive *E. coli* isolates. All *stx*-positive *E. coli* analyzed showed resistance to multiple drugs, that is, from 4 to 10 antimicrobials per clinical isolate (streptomycin, tetracycline, cephalothin, ampicillin, sulfamethoxazole + trimethoprim, nitrofurantoin and nalidixic acid, ciprofloxacin, gentamicin, and chloramphenicol). Effective management measures should be implemented, including clinical and laboratory monitoring, in order to promote animal and worker health and welfare, prevent and control the spread of diseases, and ensure effective treatment of infectious diseases. *E. uniflora* L. leaves showed inhibition of microbial growth based on the diameter of halos, ranging from 7.9 to 8.0 mm and 9.9 to 10.1 mm for concentrations of 50 and 150 mg/mL, respectively. This plant displayed bacteriostatic action and a minimum inhibitory concentration of 12.5 mg/mL for all clinical isolates. Its clinical or synergistic effects with antimicrobial agents must be determined from clinical and preclinical trials.

**Résumé**

Le but de cette étude était d'évaluer la présence d'Escherichia coli (STEC) productrices de Shiga toxine (*stx*) chez les veaux nouveau-nés diarrhéiques, ainsi que le profil de résistance de ce microorganisme aux antimicrobiens couramment utilisés en thérapie vétérinaire. Le profil antimicrobien d'Eugenia uniflora contre les isolats cliniques d’E. coli a également été analysé. Des échantillons de la muqueuse de la jonction recto-anale ont été étudiés en utilisant un milieu chromogène et l'identification d’E. coli a été effectuée à l’aide de méthodes microbiologiques (coloration de Gram, test à l’indole, test au rouge de méthyle, test de Voges-Proskauer, test à la citrate, test d’urée et production de soufre d’hydrogène). Les gènes *stx1* et *stx2* correspondant au pathotype STEC ont été évalués en utilisant la réaction en chaîne par polymérase et l'électrophorèse. Le profil de sensibilité aux agents antimicrobiens couramment utilisés dans la pratique thérapeutique vétérinaire et l'effet antimicrobien de l'extrait hydroalcohoîlique lyophilisé de feuilles d’E. uniflora L. contre les isolats cliniques d’E. coli ont été évalués par des méthodes de diffusion en disque et de microdilution. Des E. coli positifs à la Shiga toxine ont été identifiés chez 45% des veaux nouveau-nés diarrhéiques (*stx1* = 23.2 %, *stx2* = 4.0 %, *stx1* + *stx2* = 18.2 %). La fréquence des E. coli *stx*-positifs dans la population bactérienne était égale à 17,0 % (168/990 isolats cliniques) : 97 (9,8 %) E. coli positifs pour *stx1*, 12 (1,2 %) E. coli positifs pour *stx2*, et 59 isolats d’E. coli positifs pour *stx1* + *stx2* (6,0 %). Tous les E. coli *stx* positifs analysés ont montré une résistance à plusieurs médicaments, à savoir de 4 à 10 antimicrobiens par isolat clinique (streptomycine, tetracycline, céphalothine, ampicilline, sulfaméthoxazole + triméthoprime, nitrofurantoïne et acide nalidixique, ciprofloxacine, gentamicine et chloramphénicol). Des mesures de gestion efficaces devraient être mises en œuvre, y compris une surveillance clinique et de laboratoire, afin de promouvoir la santé et le bien-être des animaux et des travailleurs, de prévenir et de contrôler la propagation des maladies et de garantir un traitement efficace des maladies infectieuses. Les feuilles d’E. uniflora L. ont montré une inhibition de la croissance microbienne basée sur le diamètre des zones, allant de 7,9 à 8,0 mm et de 9,9 à 10,1 mm pour des concentrations de 50 et 150 mg/mL, respectivement. Cette plante a montré une action bactériostatique et une concentration inhibitrice minimale de 12,5 mg/mL pour tous les isolats cliniques. Ses effets cliniques ou synergiques avec les agents antimicrobiens doivent être déterminés à partir d’essais cliniques et précliniques.

(Traduit par Docteur Serge Messier)
Introduction

Brazil has been gradually increasing its rate of milk production. This rise in production demands that livestock products be produced at the highest quality, especially for exportation. Therefore, each step of milk processing is monitored, as well as the diseases that might affect the animals and impair the quality of the end product (1). Neonatal diarrhea is one of the main diseases that affects calves in their first weeks of life and compromises dairy production because of the cost of treatment and disruptions from animal loss. Among the infectious agents frequently associated with this pathology, Escherichia coli is an opportunistic Gram-negative bacillus present in the normal microbiota of the intestinal tract of humans and animals (2). This microorganism is classified into different pathotypes according to its mechanism of pathogenicity. Among these, Shiga toxin (stx)-producing E. coli (STEC) have major implications in the veterinary field. Domestic ruminants, mainly bovines, are known reservoirs of STEC, occasionally suffering from diarrhea (3).

Several studies in animals, foods, and humans have indicated the presence of STEC in Brazil; although, there are no outbreaks in the country (4). Shiga toxin (stx)-producing E. coli, however, has been related to several foodborne outbreaks worldwide. Infections caused by STEC have been reported in several South American countries, including areas in Argentina, which borders Brazil (5). Cases of antimicrobial drug resistance have been recurrent because of the indiscriminate use of antimicrobial agents, which triggers a mechanism of selection for resistant strains. Antimicrobials frequently used in veterinary therapy have a wide spectrum of action, which favors the emergence of resistance. Therefore, it becomes necessary to execute studies that evaluate the resistance profile of pathogenic bacteria and search for new strategies to fight them (6,7).

To reverse this trend, medicinal plants have been researched to discover new active principles with antimicrobial action. Given the ecological biodiversity, especially in Brazil, there is a wide range of medicinal plants to help foster exploration and scientific research and offer an interesting strategy for the control of resistant microorganisms. From the perspective of clinical and preclinical studies, potential antimicrobial effects of Eugenia uniflora L. have been reported in the literature, including:

i) Reduced adhesion of Candida albicans and Candida species to human buccal epithelial cells, reduced cell surface hydrophobicity, and decreased capacity of Candida spp. Biofilm formation (8);
ii) Antibacterial activity against methicillin-resistant Staphylococcus aureus (MRSA) (9);
iii) Antibacterial activity against S. aureus and E. coli, including synergistic or antagonistic effects when E. uniflora L. is combined with antibiotics (10);
iv) Anti-gingivitis properties and antibacterial potential against Streptococcus mutans, S. oralis, and Lactobacillus casei (11);
v) Fungicidal effect against Trichophyton rubrum, T. mentagrophytes, and clinical isolates of dermatophytes (12); and
vi) Antibacterial activity against strains isolated from clinical human specimens (MRSA and methicillin-sensitive S. aureus, E. coli, Pseudomonas aeruginosa, and Salmonella typhimurium) and food (S. enteritidis) (13).

With this in mind, the purpose of this study was to i) evaluate the occurrence of STEC in samples originating from diarrheic calves; ii) evaluate the resistance profile of this microorganism against the antimicrobials routinely used in veterinary therapy; and iii) investigate the antimicrobial action of the hydroalcoholic extract of E. uniflora L. leaves.

Materials and methods

Animals

This study involved 100 diarrheic newborn calves (Dutch, Jersey, Gir, and half-breed) between 1 and 90 d of age, belonging to 4 rural properties: A (n = 22), B (n = 28), C (n = 19), and D (n = 31). The farms, which were located near Alfenas, Minas Gerais, Brazil, all specialized in milk production and had cases of diarrheal syndrome. This research was approved by the Committee of Ethics in Research Involving Animals of Universidade José do Rosário Vellano (UNIFENAS) (CEAU Protocol no. 08A/2014).

Sampling

Microbiological samples were obtained from July 2014 to March 2015 using a previously described method (14), with some adaptations. For each diarrheic newborn calf, the samples from the recto-anal junction mucosa were harvested using a sterile swab, in the presence of a veterinarian, and maintained in 50 mL polypropylene tubes containing 10 mL of sterile phosphate-buffered saline (PBS) solution (100 mM NaCl, 100 mM NaH₂PO₄, pH = 7.2). Samples were kept at 4°C and properly transported to the Laboratory of Biology and Physiology of Microorganisms, UNIFENAS, where they were processed immediately. The tubes were centrifuged at 1700 × g for 10 min and the sediments were resuspended in 1 mL of sterile PBS solution (concentrated sample × 10). Soon after, the sediments were transferred to 2 mL microtubes and vortexed for 30 s. Then, 50 μL aliquots of each sample were inoculated on plates containing chromogenic medium (HiCrome E. coli Agar, M1295-500G; HiMedia Laboratories, Mumbai, India) and aerobically incubated at 35°C for 24 h. This medium was recommended for preliminary isolation of E. coli from foods and clinical samples (15,16). Where necessary, samples were serially diluted (1:10 to 1:10 000) to allow for more accurate counting and avoid confluence of colonies. Up to 10 colonies of greenish color were selected at random by sampling, cultured in tubes containing Brain Heart Infusion (BHI) Agar (M211-500G; HiMedia Laboratories), and kept at 37°C for 24 h to obtain pure cultures.

Identification

The bacterial isolates preliminarily identified as E. coli (i.e., greenish colonies on HiCrome agar) were characterized by microbiological methods: Gram staining (Gram-negative bacilli) and biochemical methods such as the indole test, methyl red test, Voges-Proskauer test, citrate test, urease test, and hydrogen sulfide test. Freshly cultured (BHI agar at 37°C for 24 h) E. coli isolates were stored i) in tubes containing 5 mL of BHI agar slant medium at −4°C; and ii) in tubes containing 3 mL of BHI broth medium and sterile glycerol (final concentration of 10%) at −70°C, for short and long periods,
respectively. Such procedures enabled us to access these cultures throughout the course of the analyses (17).

**DNA**

Genomic DNA of the bacterial isolates of *E. coli* were obtained using previously described methods (18). Newly grown bacterial cells were cultivated in 50 mL of BHI Medium (M211-500G; HiMedia Laboratories) at 150 revolutions per minute (Incubator shaker SL 222; SOLAB Equipamentos para Laboratórios, Piracicaba, Brazil) at 37°C for 24 h. Soon after, 1.5 mL aliquots of bacterial cells were transferred aseptically to 2 mL Eppendorf tubes and washed twice in 1 mL of PBS solution, subjecting each wash to a centrifugal force of 10 000 rpm (Centrifuge 5810R; Eppendorf do Brasil, Alto da Lapa, Brazil) for 2 min at 4°C. The cellular sediments were mixed in 500 μL Type 1 water (Milli-Q Direct 8; Millipore Indústria e Comércio, Bartueri, Brazil), agitated by vortexing for 30 s, and boiled for 20 min (Banho Ultratermostatizado Microprocessado Digital, code no. SP-152/10; SPLABOR, Presidente Prudente, Brazil). After, the sediments were centrifuged at 10 000 rpm for 2 min at 4°C and the supernatants (aliquots of 300 μL) were stored at −20°C until the moment of use.

**Primers and amplification**

The pairs of primers for genes *stx1* (forward 5'-AGA GCG ATG TTA CGG TTT CTT CGG TAT C-3' and reverse 5'-TTG CCC CCA GAG TGG ATG-3') and *stx2* (forward 5'-TGG GTT TTT CTT CGG TAT C-3', and reverse 5'-GAC ATT CTG GTT GAC TCT CTT-3') were employed to amplify DNA fragments of approximately 388 bp and 807 bp, respectively, using the following programs: 94°C for 2 min (initial DNA denaturation), 30 s (annealing), and 72°C for 30 s (elongation). The final elongation cycle was conducted at 72°C for 7 min.

**Electrophoresis**

Amplified PCR products (aliquots of 25 μL) were analyzed by electrophoresis in agarose gels at 1.8% (m/v) (UltraPure Agarose, catalog no. 16500500; Thermo Fisher Scientific), prepared with TBE buffer solution: 445 mM Tris-base, 89 mMboric acid, and 2 mM EDTA) Electrophoresis was performed in a horizontal and continuous system containing 0.5 × TBE at 150 volts for 1 h (PowerPac Universal Power Supply; Bio-Rad Laboratories, Hercules, California, USA). Positive controls (i.e., strains positive for the enterotoxins being tested) and negative controls (i.e., sterile purified water) were systematically applied in each gel next to molecular mass markers (Invitrogen 100 bp DNA Ladder, catalog no. 15628019; Thermo Fisher Scientific) in order to ensure the reproducibility of results. After the electrophoretic procedures, the gels were stained with ethidium bromide (1 μL/mL), visualized, and photodocumented (Kodak Gel Logic 200 Imaging System; Eastman Kodak Company, Rochester, New York, USA).

**Antimicrobial susceptibility test**

*Escherichia coli* isolates displaying *stx1, stx2*, or *stx1 + stx2* genes were submitted for susceptibility testing, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (20, 21). The antimicrobial susceptibility test was performed using the disk diffusion method with the following antimicrobials selected according to the therapeutic practice used in veterinary medicine: gentamicin 10 μg (GEN; aminoglycoside), streptomycin 10 μg (EST; aminoglycoside), cephalothin 30 μg (CFL; cephalosporin), chloramphenicol 30 μg (CLO; phenicol), ciprofloxacin 5 μg (CIP; fluoroquinolone), sulfamethoxazole + trimethoprim 25 μg (SUT; folate metabolism inhibitor), nitrofurantoin 300 μg (NIT; nitrofurantoin), ampicillin 10 μg (AMP; penicillin), nalidixic acid 30 μg (NAL; quinolone), and tetracycline 30 μg (TET; tetracycline) (Centro de Controle e Produtos para Diagnósticos, São Paulo, Brazil). ETEST strips (bioMérieux, São Paulo, Brazil) were also used to determine inhibitory concentration of GEN and SUT antimicrobials.

**Plant extract**

The hydroalcoholic extract was commercially purchased and stored according to the manufacturer’s recommendations (fluid extract of *E. uniflora* L. leaves; Aksy Commercial, São Paulo, Brazil). This extract was submitted to solvent removal proceedings by rotary evaporation and then lyophilized in penicillin glass vials (50 mL) (22). The final lyophilized product was prepared in an aqueous solvent (Mueller-Hinton Broth, code no. CM0405; Thermo Fisher Scientific) at analysis concentration of 2×, sterilized by filtration (Durapore Membrane Filter, 0.22 μm pore size, hydrophilic PVDF, 47 mm membrane, catalog no. GVWP04700; Millipore), and stored in sterile 50 mL polypropylene tubes at −70°C until moment of use.

**Susceptibility test and *E. uniflora* L.**

The profile of the antimicrobial susceptibility of *E. coli* isolates against *E. uniflora* L. leaves was determined by the agar diffusion method, following the guidelines established by the CLSI (20), with some adaptations. For each *E. coli* isolate, a bacterial suspension (1 to 4 × 10⁸ CFU/mL of 145 mM NaCl) was plated (spread plate method) on sterile Mueller-Hinton Agar (code no. CM0337; Thermo Fisher Scientific) previously prepared in Petri dish (150 × 15 mm; 50 mL growth medium/plate; height in each plate = 4 ± 0.5 mm) arranged in 3 equidistant wells (8 ± 1 mm of Ø). Then, 100 μL of extract solutions (50, 100, and 150 mg/mL) were poured in wells and the plates were inversely incubated at 35°C for 24 h. These tests were performed in triplicate and the interpretation of results was carried out from the zone of inhibition of microbial growth (Ø of the halo in mm). Chlorhexidine 0.12% (m/v) and Type 1 water were used as a positive control (zone of inhibition) and a negative control (lack of zone of inhibition), respectively.

The minimum inhibitory concentration (MIC) of *E. uniflora* L. leaves against *E. coli* isolates was determined by the broth microdilution method, following the guidelines established by the CLSI (21), with some adaptations. The concentrations tested covered a range of 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390, 0.195, 0.097, 0.048, 0.024, and 0.012 mg/mL. These tests were performed in triplicate, employing...
Table I. Incidence of **stx**-positive *E. coli* carriers (recto-anal junction mucosa) in diarrheic newborn calves coming from dairy farms in southern Minas Gerais, Brazil.

<table>
<thead>
<tr>
<th>Farm</th>
<th><strong>stx</strong>1</th>
<th><strong>stx</strong>2</th>
<th><strong>stx</strong>1 + <strong>stx</strong>2</th>
<th><strong>Σ</strong> (<strong>stx</strong>1)</th>
<th><strong>stx</strong>(-) <em>E. coli</em></th>
<th><strong>Σ</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>4.0</td>
<td>3</td>
<td>3.0</td>
<td>12</td>
<td>12.1</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>5.1</td>
<td>0</td>
<td>0.0</td>
<td>9</td>
<td>9.1</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>5.1</td>
<td>1</td>
<td>1.0</td>
<td>9</td>
<td>9.1</td>
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<td>9.1</td>
<td>0</td>
<td>0.0</td>
<td>13</td>
<td>13.1</td>
</tr>
<tr>
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<td>4</td>
<td>4.0</td>
<td>45</td>
<td>45.5</td>
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**Results**

**STEC and its antimicrobial susceptibility profile**

A total of 990 clinical microbial isolates were identified as *E. coli* based on microbiological methods (i.e., chromogenic medium HiCrome *E. coli* Agar, Gram stain, indole test, methyl red test, Voges-Proskauer test, citrate test, urease test, and hydrogen sulfide test). These isolates were obtained from recto-anal junction mucosa of 99 diarrheic newborn calves [i.e., 99% of sampling (about 10 isolates per diarrheic newborn calf)]. A sampling was discarded due to suspected contamination (farm D; *n* = 30).

Research on the **stx**1 and **stx**2 genes by molecular method (i.e., PCR) (Figure 1), which corresponds to the virulence factors for the STEC pathotype, identified a total of 45 (45.5%) diarrheic newborn calf carriers (recto-anal junction mucosa) of **stx**-positive *E. coli*: 23 (23.2%) with **stx**1-positive *E. coli*, 4 (4.0%) with **stx**2-positive *E. coli*, and 18 (18.2%) with **stx**1 + **stx**2-positive *E. coli* (Table I). The frequency of **stx**-positive *E. coli* in the bacterial population was equal to 17.0% (168/990) clinical isolates of *E. coli*. Of these, 97 (9.8%) were molecularly identified as **stx**1-positive *E. coli*, 12 (1.2%) as **stx**2-positive *E. coli*, and 59 (6.0%) as **stx**1 + **stx**2-positive *E. coli* (Table II).

The antimicrobial susceptibility of 34 (20.2%) clinical isolates, randomly selected from the population of clinical specimens and identified as of **stx**-positive *E. coli* (*n* = 168), was analyzed according to the CLSI guidelines (20) using antimicrobials AMP, CFL, CLO, CIP, GEN, NAL, NIT, EST, SUT, and TET. Among the **stx**-positive *E. coli* isolates, high resistance and/or intermediate resistance to antimicrobials was observed: 34 (100%) were EST<sup>R</sup>, 33 (97.1%) were TET<sup>R</sup>, 33 (97.1%) were CFL<sup>R</sup> [i.e., 30 CFL<sup>R</sup> (88.2%) and 3 CFL<sup>I</sup> (8.8%)], 32 (94.2%) were AMP<sup>R</sup> [i.e., 28 AMP<sup>R</sup> (82.4%) and 4 AMP<sup>I</sup> (11.8%)], 30 (88.2%) were SUT<sup>R</sup>, 26 (76.5%) were NIT<sup>R</sup> and NAL<sup>R</sup> [i.e., 25 NAL<sup>R</sup> (73.5%) and 1 NAL<sup>I</sup> (2.9%)], 23 (67.6%) were CIP<sup>R</sup> [i.e., 14 CIP<sup>R</sup> (41.2%) and 9 CIP<sup>I</sup> (26.5%)], 22 (64.7%) were GEN<sup>R</sup> [i.e., 18 GEN<sup>R</sup> (52.9%) and 4 GEN<sup>I</sup> (11.8%)], and 16 (47.1%) were CLO<sup>R</sup> [i.e., 11 CLO<sup>R</sup> (32.4%) and 5 CLO<sup>I</sup> (14.7%)] (Table III).

Of these 30 **stx**-positive *E. coli* isolates SUT<sup>R</sup>, all isolates (100%) showed absence of inhibition ellipse by the Etest method. Of these 22 **stx**-positive *E. coli* isolates GEN<sup>R</sup>, 2 and 20 isolates were considered sensible and resistant to GEN, respectively, by the Etest.
method: 1 (4.5%) isolate GENI showed MIC value of 0.75 μg/mL, 1 (4.5%) isolate GENI showed MIC value of 1.5 μg/mL, 2 (9.1%) isolates GENI showed MIC values of 24 μg/mL, 1 (4.5%) isolate GENR showed MIC value of 38 μg/mL, 2 (9.1%) isolates GENR showed MIC values of 48 μg/mL, 4 (18.2%) isolates GENR showed MIC values of 64 μg/mL, and 11 (50.0%) isolates GENR showed absence of inhibition ellipse.

### STEC and its susceptibility profile to E. uniflora L.

The susceptibility profile of stx-positive E. coli isolates against lyophilized hydroalcoholic extract of E. uniflora L. leaves, which was determined by the agar diffusion method, showed inhibition halos with diameters of 7.9 to 8 mm, 9 mm, and 9.9 to 10.1 mm for concentrations of 50 mg/mL, 100 mg/mL, and 150 mg/mL, respectively. The MIC of E. uniflora L. against E. coli clinical isolates, which was determined by the broth microdilution method, was equal to 12.5 mg/mL for all isolates analyzed. The action of this extract was considered bacteriostatic, as determined by the MBC assay (Table III).

### Discussion

#### Multi-drug resistant STEC

This research presented a high frequency of E. coli isolated from recto-anal junction mucosa of diarrheic newborn calves (99%). Almost half of these diarrheic newborn calves (45%) carried stx-positive E. coli. However, a low frequency (17%) of stx-positive E. coli was observed in the bacteria population studied, the stx1 allele being more frequently observed than the stx2 allele. These bacteria showed resistance to multiple drugs (from 4 to 10 antimicrobials per resistant clinical isolate based on data of resistant and intermediate), especially EST followed by TET, CFL, AMP, SUT, NIT and NAL, CIP, GEN, and CLO:

- 2 (5.9%) E. coli clinical isolates resistant to 4 antimicrobials;
- 1 (2.9%) E. coli clinical isolate resistant to 5 antimicrobials;
- 4 (11.8%) E. coli clinical isolates resistant to 6 antimicrobials;
- 4 (11.8%) E. coli clinical isolates resistant to 7 antimicrobials;
- 5 (14.7%) E. coli clinical isolates resistant to 8 antimicrobials;
- 10 (29.4%) E. coli clinical isolates resistant to 9 antimicrobials; and
- 8 (23.5%) E. coli clinical isolates resistant to 10 antimicrobials.

Bovines are known for being asymptomatic reservoirs of STEC and represent an important source of infection. Several studies have been conducted in order to understand the STEC pathogenesis mechanism and search for new ways to suppress future infections. Many findings in the literature on STEC frequency corroborate our data from this study (25-28), including in some regions of Brazil, such as Paraná (27), Minas Gerais (29), Rio de Janeiro and Rondônia (25), and São Paulo (28). However, such frequencies may be different in other regions. For example, the investigation of the virulence genes in E. coli isolated from dairy cows in São Paulo, Brazil shows frequencies of 16%, 17.3%, and 6.6% for genes stx1, stx2, and stx1 + stx2, respectively (30). A study on STEC O157 in feces at dairy cattle farms in South Africa shows frequencies of 44%, 45.3%, and 10.7% for genes stx1, stx2, and stx1 + stx2, respectively (31). The research on STEC in fecal samples of crossbred finishing cattle harvested from pens in a commercial feedlot in the central United States reveals a high frequency of genes stx2 (94.1%) and stx1 (64.4%) (32). The variability in the occurrence of STEC in clinically healthy animals (high and low frequencies) and animals with signs of infection (mainly at high frequency) has been reported especially in the midwest, southwest, and south regions of Brazil, according to recently published review data (33). In addition, Brazilian STEC carrying the stx1 gene was more frequently found in cattle, whereas STEC with the stx2 gene was prevalently observed in food and their virulence profiles suggested potential pathogenicity to humans (33).

As the incidence of STEC strains has been high in animals with diarrhea, it is necessary to evaluate antimicrobial susceptibility profiles to ensure therapeutic success. In addition, several studies in veterinary therapy have reported increasing concerns due to cases of antibiotic resistance in STEC. For example, STEC strains isolated from fecal samples of diarrheic calves showed antimicrobial resistance to multiple drugs, such as penicillin, ETP, TET, lincomycin, CLO, AMP, trimethoprim, enrofloxacin, and CIP (34). Shiga toxin-producing E. coli strains O157 and non-O157 isolated from cattle, sheep, and poultry also revealed a predominant resistance to AMP, CFL, CLO, and kanamycin (35). Shiga toxin-producing E. coli strains isolated from dairy cows displayed high levels of resistance to AMP, TET, trimethoprim, erythromycin, NAL, EST, novobiocin, lincomycin, penicillin, and neomycin. Additionally, the dissemination of multidrug resistant strains can lead to several implications for public health, since it can hamper treatment (30). Shiga toxin-producing E. coli strains isolated from fecal samples of dairy cattle showed a high prevalence of multidrug resistances, including AMP, TET, oxytetracycline, amoxicillin + clavulanate, CFL, ceftazidime, norfloxacin, CIP, enrofloxacin, amikacin, CLO, kanamycin, EST, GEN, and SUT (31). In addition, the resistance profile may vary among the farms and the management conditions can influence the incidence of resistant strains (36).
Table III. Antimicrobial susceptibility profiles and minimal inhibitory concentration of *E. uniflora* L. of the stx-positive *E. coli* clinical isolates (recto-anal junction mucosa) of diarrheic newborn calves coming from dairy farms in southern Minas Gerais, Brazil. A, B, and C correspond to concentrations of 50, 100, and 150 mg/mL of lyophilized hydroalcoholic extract of *E. uniflora* leaves, respectively.

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<thead>
<tr>
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GEN — gentamicin; EST — streptomycin; CFL — cephalothin; CLO — chloramphenicol; CIP — ciprofloxacin; SUT — sulfamethoxazole + trimethoprim; NIT — nitrofurantoin; AMP — ampicillin; NAL — nalidixic acid; TET — tetracycline; MIC — minimal inhibitory concentration; R — resistant; I — intermediate; S — susceptible.
Effect of *E. uniflora* leaves on STEC

The elevated incidence of multidrug resistant STEC and non-STEC strains has been prompting the search for new antimicrobial agents that are efficient against these increasingly resistant strains. Therefore, several studies with medicinal plants have been performed in order to find new bioactive principles, especially with potential antimicrobial properties capable of containing multidrug resistant strains. In this study, the susceptibility of six-positive *E. coli* isolates to lyophilized hydroalcoholic extract of *E. uniflora* L. leaves was tested. The inhibition of microbial growth was observed on culture medium (agar diffusion method) by producing inhibition halos of varying sizes (7.9 to 10.1 mm) depending on the extract concentrations (50 to 150 mg/mL). The MIC of *E. uniflora* L. against all *E. coli* isolates tested was 12.5 mg/mL and its action was considered bacteriostatic. The minimal bactericidal and fungicidal concentrations from *E. uniflora* extracts have been considered to be absent (37) or within a twofold dilution of the MIC (38) for some microorganisms, including *E. coli* species.

A previous investigation has contributed to the knowledge of antimicrobial properties of plants commonly found in Brazil, including the species *E. uniflora* L. (37). The ethanolic extract of *E. uniflora* L. leaves revealed antimicrobial and anti-biofilm activities. Planktonic cells (reference strains: *S. aureus*, *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, *C. albicans*, *C. parapsilosis*, and *C. tropicalis*) and biofilm (*C. albicans*) displayed MICs of 31.2 to 125 µg/mL (yeasts), >1000 µg/mL (bacteria), and 1000 µg/mL (biofilm) (37). A clinical isolate of *E. coli* EC27 (resistant to neomycin, GEN, tobramycin, amikacin, and kanamycin), and *E. coli* ATCC 8559 were also tested against the ethanolic extract of *E. uniflora* leaves. Antimicrobial activity of the extract was not found at 1024 mg/mL, but a synergism occurred between *E. uniflora* and GEN, leading to a reduction of the MIC to GEN. This result showed that *E. uniflora* L. could also be used as a source of natural products capable of enhancing antimicrobial action (39).

In a study involving spore-forming and non-sporing Gram-positive and Gram-negative bacteria, and yeast (reference strains: *Micrococcus roseus* ATCC 1740, *M. luteus* ATCC 9341, *B. cereus* ATCC 14576, *B. stearothermophilus* ATCC 1262, *B. subtilis* ATCC 6633, *Enterobacter aerogenes* ATCC 13048, *E. coli* ATCC 11229, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *P. aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 14756, *S. aureus* ATCC 6538, *S. aureus* ATCC 25923, and *S. epidermidis* ATCC 12228; others strains: *C. albicans* NTC 2010, *S. aureus* 481, *E. cloacae* HMA/FTA 502, *E. coli* 8739, and *S. aureus* 897, 912, 934, 934 and 937), the MIC of the crude ethanol extract of *E. uniflora* L. leaves ranged from 0.273 mg/mL to 35 mg/mL, depending on the microbial species. The MIC of *E. uniflora* L. for *E. coli* was 17.5 mg/mL. These antimicrobial activities of *E. uniflora* L. may be due to the presence of flavonoids and tannins in its chemical constitution (40). The antimicrobial activity of the ethanol extract of *E. uniflora* L. fruits and other anatomical parts of Brazilian plants was tested with the disk diffusion method using some clinical microbial isolates. Microbial growth inhibition (i.e., inhibition halo) was observed for species of *E. coli* (intra-abdominal infection), *S. pyogenes* (oropharynx infection), *Providencia* spp. (wound), *Proteus mirabilis* (intra-abdominal infection), *Shigella sonnei* (feces), *S. aureus* (infection), and negative coagulase *Staphylococcus* spp. (conjunctivitis secretion). Considering the importance of medicinal plants, this knowledge will possibly contribute to the well-being of developing countries, both as a therapeutic resource and an economic resource (41).

The antimicrobial activity and MIC evaluation of the ethanol extract of *E. uniflora* L. leaves and other anatomical parts of Brazilian plants was also tested by microdilution methods using the reference strains of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, *B. subtilis* ATCC 6623, *S. aureus* ATCC 25923, and some clinical isolates of *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. The MIC values ranged from 250 µg/mL to >1000 µg/mL for bacteria, with the MIC for *E. coli* being equal to 500 µg/mL and the MIC for yeasts between 31.2 µg/mL and >1000 µg/mL. The results may justify the ethnobotanical use of this and other plant species in the treatment of infectious diseases (38).

The variability of the antimicrobial response of *E. uniflora* L. may occur, at least in part, due to factors such as geographic location, anatomy, climate, altitude, soil composition, harvest time, development stage, seasonality, as well as laboratory methods for extracting and obtaining bioactive compounds. The sum of these factors can qualitatively and quantitatively affect the plant metabolism and the bioactive compound produced. In addition, the production of potentially therapeutic and medicinal plants is dependent on genetic background and environmental conditions. Their major bioactive compounds may vary according to different environments due to responses to different physical, chemical, and biotic elicitors (42).

In conclusion, this research potentially adds to existing epidemiological data on multi-drug resistant bacteria, especially STEC associated with diarrheal syndrome in newborn calves from dairy farms. The frequency of potentially virulent and antimicrobial resistant *E. coli* strains found in this study points to the need for appropriate animal management measures, including ongoing clinical and laboratory monitoring, in order to promote animal and worker health and welfare, prevent and control the spread of diseases, and effectively treat infectious diseases.

The growing body of evidence on resistant strains to known antimicrobials, coupled with the fact that many of these antimicrobials have harmful side effects, leads to the need for research to find new substances that are effective in treating infections. This study contributes to information about the antimicrobial potential of the lyophilized hydroalcoholic extract of *E. uniflora* L. leaves against STEC clinical isolates coming from recto-anal junction mucosa of diarrheic newborn calves. This plant showed inhibition of microbial growth, the same MIC for all clinical isolates (12.5 mg/mL), besides its bacteriostatic action. However, the evaluation of the potentially synergistic effect of this extract with antimicrobial agents used in infectious treatments must be determined from clinical and preclinical trials.

Acknowledgment

This research was supported by Rede Mineira de Ensaios Toxicológicos e Farmacológicos de Produtos Terapêuticos (REDE MINEIRA TOXIFAR), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG processo no. RED-0008-14).
References


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Antimicrobial resistance and recovery of *Salmonella*, *Campylobacter*, and *Escherichia coli* from chicken egg layer flocks in Canadian sentinel surveillance sites using 2 types of sample matrices

Agnes Agunos, Sheryl P. Gow, David F. Léger, Logan Flockhart, Danielle Daignault, Andrea Desruisseau, Erin Zabek, Frank Pollari, Richard J. Reid-Smith

**Abstract**

Eggs are important to the diet of Canadians. This product is one of the supply-managed commodities in Canada, but unlike other commodities, where food safety risks are extensively explored and reported, information on the prevalence of enteric organisms (e.g., *Salmonella*, *Campylobacter*) and antimicrobial resistance (AMR) in layers in Canada are limited. This study was conducted to determine the prevalence of select bacteria and the associated AMR patterns in layer flocks using 2 sample matrices. Farms were located within FoodNet Canada and the Canadian Integrated Program for Antimicrobial Resistance Surveillance sentinel sites (SS). Fecal samples (Ontario: ON<sub>SS1a</sub>, ON<sub>SS1b</sub>) and environmental sponge swabs (British Columbia: BC<sub>SS2a</sub>) were collected. *Salmonella* prevalence was 29% and 8% in ON<sub>SS1a</sub> and ON<sub>SS1b</sub> respectively, and 7% in BC<sub>SS2a</sub>. *S. Kentucky* and *S. Livingstone* were the most frequently isolated serovars and no *S. Enteritidis* was detected. *Campylobacter* was not detected in the BC sponge swabs but was isolated from 89% and 53% of Ontario fecal samples (ON<sub>SS1a</sub> and ON<sub>SS1b</sub> respectively). Seven *C. jejuni* from Ontario were ciprofloxacin-resistant. *Escherichia coli* prevalence was high in both sample types (98%). Overall, tetracycline resistance among *E. coli* ranged from 26% to 69%. Resistance to ceftiofur (n = 2 isolates) and gentamicin (n = 2) was relatively low. There were diverse resistance patterns (excludes susceptible isolates) observed among *E. coli* in Ontario (10 patterns) and British Columbia (14 patterns). This study revealed that fecal samples are more informative for farm-level monitoring of pathogen and AMR prevalence. Without further validation, sponge swabs are limited in their utility for *Campylobacter* detection and thus, for public health surveillance.

**Résumé**

Les œufs sont importants pour l'alimentation des Canadiens. Ce produit est l’un des produits soumis à la gestion de l’offre au Canada, mais contrairement à d’autres produits, où les risques pour la salubrité des aliments sont largement étudiés et signalés, des informations sur la prévalence des organismes entériques (p. ex. *Salmonella*, *Campylobacter*) et la résistance aux antimicrobiens (RAM) chez les pondeuses au Canada sont limitées. Cette étude a été menée pour déterminer la prévalence de certaines bactéries et les patrons de résistance aux antimicrobiens associés dans les troupeaux de pondeuses en utilisant deux matrices d’échantillons. Les fermes étaient situées au sein de FoodNet Canada et des sites sentinelles (SS) du Programme intégré canadien de surveillance de la résistance aux antimicrobiens. Des échantillons de matières fécales (Ontario : ON<sub>SS1a</sub>, ON<sub>SS1b</sub>) et des éponges environnementales (Colombie-Britannique : BC<sub>SS2a</sub>) ont été prélevés. La prévalence de *Salmonella* était de 29 % et 8 % pour ON<sub>SS1a</sub> et ON<sub>SS1b</sub> respectivement, et de 7 % pour BC<sub>SS2a</sub>. *S. Kentucky* et *S. Livingstone* étaient les sérotypes les plus fréquemment isolés et aucun *S. Enteritidis* n’a été détecté. *Campylobacter* n’a pas été détecté dans les éponges de la Colombie-Britannique, mais a été isolé de 89 % et 53 % des échantillons de matières fécales de l’Ontario (ON<sub>SS1a</sub> et ON<sub>SS1b</sub> respectivement). Sept *C. jejuni* de l’Ontario étaient résistants à la ciprofloxacine. La prévalence d’*Escherichia coli* était élevée dans les deux types d’échantillons (98 %). Dans l’ensemble, la résistance à la tétracycline chez *E. coli* variait de 26 % à 69 %. La résistance au ceftiofur (n = 2 isolats) et à la gentamicine (n = 2) était relativement faible. Divers profils de résistance (à l’exclusion des isolats sensibles) ont été observés chez *E. coli* en Ontario (10 profils) et en Colombie-Britannique (14 profils). Cette étude a révélé que les échantillons fécaux sont plus informatifs pour la surveillance au niveau de la ferme de la prévalence des agents pathogènes et de la résistance aux antimicrobiens. Sans validation supplémentaire, les éponge sont limitées dans leur utilité pour la détection de *Campylobacter* et donc pour la surveillance en santé publique.

(Traduit par Docteur Serge Messier)
**Introduction**

In Canada, the per capita consumption of eggs in 2018 was 21 dozen per person, indicating that this commodity is important to the diet of Canadians (1). Historically, in Canada, the consumption of ungraded, Grade B, or undercooked eggs and the handling of raw eggs have been identified as risk factors for salmonellosis in humans (2–4). An industry-led initiative, Start Clean–Stay Clean (SC-SC) is the Canadian egg industry’s on-farm food safety program designed to ensure the quality and safety of table eggs and egg products. It has a protocol for the reduction of *Salmonella* prevalence, in particular, *S. Enteritidis*, which is a pathogen associated with egg-related salmonellosis outbreaks (5,6). However, there are no publicly accessible national surveillance programs showing trends in prevalence of *Salmonella*, *Campylobacter*, and other pathogens and their antimicrobial resistance (AMR) profiles in laying hens or table eggs. *Campylobacter* (447.23 cases per 100,000 population) and non-typhoidal *Salmonella* (269.26 cases per 100,000 population) ranked 3rd and 4th, respectively, among the domestically acquired foodborne illnesses in Canada (7). Within the Public Health Agency of Canada, FoodNet Canada (FNC) and Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) conduct surveillance of foodborne bacteria (e.g., *Salmonella*, *Escherichia coli*, and *Campylobacter*), from farm to fork. The FNC program is a multi-partner, sentinel-based comprehensive surveillance program that collects data in multiple sectors (e.g., agriculture and human health units) for the effective evaluation and development of policies for the safety of food and water in Canada (8). The CIPARS program monitors trends in AMR and antimicrobial use (AMU) in humans and animals and has active farm AMU and AMR surveillance programs in broiler chickens, turkeys, swine, and beef cattle (9). The collection of AMU information at the farm level complements AMR data, but there is a knowledge gap in terms of AMU in the layer sector in Canada. Although, we hypothesize that AMU is relatively low in the major egg-producing provinces such as Ontario (10) and Quebec (11), due to the relatively stable number of diagnoses of reproductive disorders in layers that have bacterial etiology, including bacterial peritonitis or salpingitis, and early systemic bacterial infections.

The CIPARS program previously conducted 2 research studies in the layer sector to determine *Salmonella* baseline prevalence and explore potential areas in the egg production chain where routine national monitoring of *Salmonella* and AMR could be implemented (12). The first study involved cecal sample collection from spent hens at the processing plant level (2009 to 2011) in Ontario, where 42% (117/279) of samples tested *Salmonella*-positive; of these 117 isolates, 15 *S. Enteritidis* were identified. The second study involved sampling liquid whole eggs at the Ontario egg-breaking stations, where 2% (5/300) of samples tested *S. Enteritidis*-positive (12). Similarly, a research project in Alberta layer flocks detected *Salmonella* in 57% and 60% of samples from barns and flocks, respectively (13).

Farm-level monitoring programs in layers such as Canada’s SC-SC program and the United States Food and Drug Administration’s 21 CFR Parts 16 and 118 “Prevention of *Salmonella Enteritidis in Shell Eggs During Production, Storage and Transportation; Final Rule” (14) aim to eliminate *S. Enteritidis* to offset the burden of illness associated with the consumption of egg and egg products, an important dietary source of protein in North America. In Canada, the annual incidence rate for endemic *S. Enteritidis* cases has remained stable, between 8.6 and 8.3 cases per 100,000 population between 2014 and 2017 (8). While human cases attributable to chicken meat, chicken-derived products, and manure have been extensively investigated by both CIPARS and FNC, data related to the egg layer sector is sparse. Globally, up to 24% of non-typhoidal *Salmonella* spp. cases have been attributed to the consumption of eggs (16). Similarly, for *Campylobacter*, up to 4.5% of human cases have been attributed to the consumption of eggs (16,17). The public health significance described above and the potential co-occurrence of these 2 major foodborne pathogens in layer flocks (18) emphasizes the importance of including the egg sector in comprehensive surveillance programs with One Health themes such as CIPARS and FNC.

The SC-SC program utilizes environmental sponge swabs (5), but the collection of manure in addition to egg belt swabs or dusts were cited as the preferred sample type in the United States Final Rule (14), as informed by routine monitoring protocols (15), and in the European Union member states (18), to enhance the diversity of the sample matrix for improved bacterial recovery. In the context of the global and Canadian public health implications of *Salmonella* and *Campylobacter* described earlier, and in an effort to align with international surveillance programs for pathogen recovery and AMR, the objectives of this study were to i) evaluate the utility of 2 different sample matrices (fecal droppings (i.e., standard farm samples used by FNC and CIPARS) and environmental sponge swabs (i.e., SC-SC program)) for the simultaneous detection of *Salmonella*, *Campylobacter*, and *E. coli* (the latter as an indicator organism routinely used for AMR monitoring); and ii) describe the resistance of these bacteria to antimicrobials used in veterinary and human medicine. This study will inform the development of surveillance protocols for layer AMR and pathogen prevalence and explore the utility of an existing monitoring platform, the SC-SC program, as a mechanism for collecting AMU data and samples for pathogen recovery and AMR testing.

**Materials and methods**

To complement *Salmonella* prevalence and AMR information previously collected by CIPARS and FNC, a farm-level pilot study in layers was conducted from sentinel sites: i) Ontario sentinel site 1a (ONSS1a) in 2013; ii) British Columbia sentinel site 2a (BCSS2a) between 2013 and 2014; and iii) Ontario sentinel site 1b (ONSS1b) between 2016 and 2017. The sampling of layer flocks was part of the larger FNC sentinel site program, which has human, environment/water, and animal commodity components (8). The priority animal commodities sampled were based on the agricultural profile of the province where the sentinel site is located. Layer flocks constituted the agricultural profile of the FNC sentinel sites above. With the support of the 2 provincial egg-marketing boards, a notification was sent to all producers explaining the study and encouraging them to support the pilot study. Participation to the study was voluntary.

The farm sampling methods are summarized in Table 1. All samples were collected during the laying phase that coincided with the sampling/flock audit schedule according to the Egg Farmers of
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<td>Specified surfaces (egg conveyor belts, manure belts, dusts)</td>
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<td>Sample preparation</td>
<td>Fecal sample + BPW (1:10 ratio), incubated at 35°C ± 1°C for 24 h</td>
<td>Pooled sponges + 100 mL BPW, incubated at 35°C ± 1°C for 24 h</td>
</tr>
</tbody>
</table>

**Table I. Summary of farm sampling protocol and microbiological methods in 2 sample matrices.**

<table>
<thead>
<tr>
<th><strong>Salmonella</strong></th>
<th><strong>Ontario</strong></th>
<th><strong>British Columbia</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• 0.1 mL BPW mixture onto MSRV; 42°C ± 1°C for 24 to 72 h incubation</td>
<td>1 mL BPW to 9.9 mL RVS broth; 42°C ± 2°C for 24 to 20 h incubation</td>
<td>Streaked to XLT4 and BGA; 35°C ± 2°C for 18 to 48 h incubation</td>
</tr>
<tr>
<td>• Migration ≥ 20 mm were streaked onto MacConkey agar</td>
<td>• Subculture: 4 Salmonella-like colonies to BAP; 35°C ± 2°C for 18 to 48 h incubation</td>
<td>• Further testing: indole (neg), oxidase (pos), Gram stain; confirmed one suspect colony using API 20E followed by serogrouping</td>
</tr>
<tr>
<td>• Further testing: TSI and urea agar slants inoculation, indole test (negative), slide agglutination with Salmonella Poly A-1 and Vi antiserum</td>
<td></td>
<td>• 2 isolates stored in cryovials (−80°C) sent to OIE Salmonella Reference Laboratory at Guelph</td>
</tr>
<tr>
<td>• All isolates sent to OIE Salmonella Reference Laboratory at Guelph</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**OIE Salmonella Reference laboratory (all isolates):** Detection of O or somatic antigens via slide agglutination; H or flagellar antigens identified with a microtitre plate well precipitation method (9).

<table>
<thead>
<tr>
<th><strong>Escherichia coli</strong></th>
<th><strong>Ontario</strong></th>
<th><strong>British Columbia</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• 0.1 mL of the BPW mixture onto MacConkey agar; 35°C ± 1°C for 18 to 24 h incubation</td>
<td>0.1 mL of the BPW mixture onto MacConkey agar; 35°C ± 1°C for 18 to 24 h incubation</td>
<td></td>
</tr>
<tr>
<td>• Lactose-fermenting colonies were screened for purity (Luria-Bertani agar); Presumptive E. coli colonies were screened for purity (TSA with 5% sheep blood)</td>
<td>2 lactose-fermenting colonies from MacConkey were subcultured in BAP</td>
<td>Stored in cryovials (−80°C) and sent to AMR Laboratory, NML at Guelph</td>
</tr>
<tr>
<td>• Further testing: Simmons citrate and indole tests; confirmed using a bacterial identification test kit (API 20E)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Campylobacter</strong></th>
<th><strong>Ontario</strong></th>
<th><strong>British Columbia</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• 1 mL BPW mixture mixed with 9 mL of HEB; 35°C ± 1°C for 4 h microaerophilic incubation</td>
<td>1 mL BPW mixed with 9 mL Enriched Bolton Broth; 35°C ± 2°C for 4 h then 42°C ± 2°C overnight incubation</td>
<td>Streaked onto CCDA; 42°C ± 2°C for 48 h microaerophilic incubation</td>
</tr>
<tr>
<td>• 36 μL sterile cefoperazone added to HEB tubes; 42°C ± 1°C for 20 to 24 h microaerophilic incubation</td>
<td></td>
<td>Subculture: at least 2 suspect colonies to Columbia BAP; 42°C ± 2°C for 24 h microaerophilic incubation</td>
</tr>
<tr>
<td>• Culture inoculated onto mCCDA plate; 42°C ± 1°C for 24 to 72 h microaerophilic incubation</td>
<td></td>
<td>Further testing: Gram stain, oxidase, and catalase</td>
</tr>
<tr>
<td>• Further testing: Gram stain, oxidase, and catalase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NML at St.Hyacinthe:** Speciation using multiplex PCR (31) with QIaxcel Advanced. This system uses capillary electrophoresis to automate analysis of DNA. It provides accurate data regarding their sizes and quantities. Any contaminating DNA fragments such as non-specific amplicons, primer–dimers, or undigested DNA are detected (9).

**Amplification of specific genomic targets from bacterial lysates:**

**CC18F:** 5′-AGG TAT GAT TTC TAG AAG CGA-3′

**CC519R:** 5′-ATA AAA GAC TAT CGT CGC GTG-3′

**Hippo-F:** 5′-GAC TTC GTTG CAG ATA TGG ATG CTT-3′

**Hippo-R:** 5′-GCT ATA ACT ATC CGA AGA AGC CAT CA-3′

**16S-F:** 5′-GGA GGC AGC AGT AGG GAA TA-3′

**16S-R:** 5′-TGA CGG GCG GTG AGT ACA AG-3′
Canada’s SC-SC program (5). The SC-SC program requires 2 sampling visits during the laying phase: 1 early lay (19 to 35 wk) and 1 late lay (36 to 60 wk).

**FNC and CIPARS sampling methodology**

In ONSS1a and ONSS1b, FNC and CIPARS requested the collection of extra fecal samples during the regular SC-SC farm visits by the field worker/Egg Farmers of Ontario staff. To harmonize with the other poultry farm surveillance programs, in which one pooled fecal sample is required for one quadrant of a barn (or sampling unit), the layer collection approach was modified depending on the structure of the laying barn (e.g., one pooled fecal sample collected from one tier of cages, one manure belt or the equivalent of a quarter of the barn population). Approximately 60 g or half of the 129 mL standard fecal containers per pooled sample was collected. For each farm, one sampling unit (barn, floor, pen) was randomly selected for testing so that each farm sample submission (n = 4 per farm) represented a single age group of layers. Field workers were instructed to collect samples from safe and accessible areas where there was obvious pooling of fecal material such as the end of the manure conveyor belts. All Ontario samples were sent to the National Microbiology Laboratory (NML) in Saint-Hyacinthe for bacterial isolation.

**SC-SC protocol**

In BCSS2a, the environmental swab samples collected for the SC-SC program were pooled in sterile Whirl-Pak bags and labelled to indicate that they were for the FNC and CIPARS enhanced testing. One pooled environmental sponge swab sample represented multiple swab locations within the barn (e.g., manure belts, egg conveyor belts, dust from walls and floors). The number of samples submitted was proportional to the bird population and varied from one (collected from 5 000 birds of the same age on the same floor) to 7 samples (collected from 35 000 birds of different ages) per farm. Flocks of the same age but from different barns were sampled separately. Each pooled sample represented one age group, thus, some of the farm submissions constituted multiple flocks. Samples were submitted to the British Columbia Ministry of Agriculture Animal Health Center (AHC) in Abbotsford, for bacterial isolation.

In both provinces, a 1-page survey sheet was included with each sampling kit/submission in order to collect farm information (e.g., date of sample collection, age of the flock(s) sampled, and total bird population) for both FNC and CIPARS. No farm identifiers were included in the submission form; the codes were kept by the Egg Farmers of Ontario and the British Columbia Egg Marketing Board staff.

**Microbiological methods**

Table I outlines the respective primary isolation steps. The British Columbia AHC forwarded all isolates to either NML in Saint-Hyacinthe (Campylobacter) or NML in Guelph (Salmonella and *E. coli*) for further characterization and susceptibility testing, per routine CIPARS protocol (9). Briefly, for *Salmonella* and *E. coli*, isolates were tested using the CMV2AGNF (2013 isolates) or CMV3AGNF (2014 to 2017 isolates) (Sensititre; Trek Diagnostic Systems, West Sussex, England), a public health panel comprised of 14 antimicrobials designed by the United States National Antimicrobial Resistance Monitoring Program (NARMS). For *Campylobacter*, isolates were tested using the CAMPY AST Plate (Sensititre; Trek Diagnostic Systems) comprised of 9 antimicrobials.

**Data analysis**

Analysis was performed with SAS 9.3 (SAS Institute, Cary, North Carolina, USA) as per routine CIPARS analysis described elsewhere (9). Data were dichotomized for each organism at the isolate level, into susceptible (including intermediate susceptibility) or resistant, using Clinical Laboratory Standards Institute (CLSI) breakpoints (20,21). In the absence of CLSI interpretative criteria, breakpoints were based on the distribution of the minimum inhibitory concentrations and harmonized with those of the NARMS (9).

As previously described, multiple samples per flock were accounted for, clustering in the calculation of prevalence estimates using generalized estimating equations with a binary outcome, logit-link function, and exchangeable correlation structure. Null binomial response models were run for each antimicrobial and organism, and from each null model, the intercept (β0) and the 95% confidence interval (CI) were used to calculate population-averaged prevalence estimates using the formula:

\[
[1 + \exp(-\beta_0)]^{-1}
\]

(9).

**Limitations of the study**

We caution our readers that the total flocks sampled were lower than the number of flocks per sentinel site routinely sampled in

---

Table I. Summary of farm sampling protocol and microbiological methods in 2 sample matrices (continued).
Table II. Recovery of *Escherichia coli*, *Salmonella*, and *Campylobacter* in layers from Canadian Integrated Program for Antimicrobial Resistance Surveillance, and FoodNet Canada sentinel sites (2013 to 2017).

<table>
<thead>
<tr>
<th>Sample matrix and sentinel site</th>
<th>Year(s)</th>
<th>Escherichia coli&lt;sup&gt;a&lt;/sup&gt; % (positive/total submitted)</th>
<th>Salmonella % (positive/total submitted), serovars (n)</th>
<th>Campylobacter % (positive/total submitted), species (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal samples (ON&lt;sub&gt;SS1a&lt;/sub&gt;)</td>
<td>2013</td>
<td>Not done</td>
<td>29% (11/38)</td>
<td>89% (34/38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Braenderup (3)</td>
<td>C. coli (n = 20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heidelberg (1)</td>
<td>C. jejuni (n = 14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kentucky (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ohio var. 14 + (2)</td>
<td></td>
</tr>
<tr>
<td>Fecal samples (ON&lt;sub&gt;SS1b&lt;/sub&gt;)</td>
<td>2016 to 2017</td>
<td>98% (59/60)</td>
<td>8% (5/60)</td>
<td>53% (32/60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kentucky (1)</td>
<td>C. coli (n = 12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Livingstone (4)</td>
<td>C. jejuni (n = 20)</td>
</tr>
<tr>
<td>Environmental sponge swab (BC&lt;sub&gt;SS2a&lt;/sub&gt;)</td>
<td>2013 to 2014</td>
<td>98% (53/54)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7% (4/54)</td>
<td>0/54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Infantis (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liverpool (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Livingstone (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mbandaka (1)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Generic *E. coli* isolates were not further characterized. All isolates were non-hemolytic.

<sup>b</sup> 2 isolates per pooled environmental sample were submitted for susceptibility testing, except for 1 sample where only 1 isolate was available for testing (N = 107).

<sup>c</sup> Antimicrobial susceptibility testing was not done on these isolates (transition year for the methodological and operational harmonization between CIPARS and FoodNet Canada farm components).

**Results**

**Flock characteristics**

The average flock population had 12 612 layer birds and ranged from 3200 to 32 000 layer birds. The average age of sampling was 37 wk and ranged from 20 to 64 wk of age.

**Recovery**

There were 38 pooled fecal samples from 9 layer farms in ON<sub>SS1a</sub> and 60 fecal samples from 15 layer farms in ON<sub>SS1b</sub> and 54 environmental sponge swab samples from 26 layer farms in BC<sub>SS2a</sub>. The *E. coli* recovery rate was comparable, 98% for both fecal and environmental sponge swabs (Table II). The *Salmonella* and *Campylobacter* recovery rate varied: 29% in ON<sub>SS1a</sub> and 8% in ON<sub>SS1b</sub> fecal samples compared to 7% in BC<sub>SS2a</sub> for *Salmonella*; 89% (ON<sub>SS1a</sub>) and 53% (ON<sub>SS1b</sub>) for the fecal samples compared to 0% for the environmental sponge swabs (BC<sub>SS2a</sub>) for *Campylobacter*.

Overall, 8 different *Salmonella* serovars were detected (Table II), the 2 most frequent being *S. Kentucky* (6 isolates) and *S. Livingstone* (5 isolates). There was no *S. Enteritidis* recovered.
Antimicrobial resistance

*Escherichia coli* data for the ON<sub>SS1a</sub> isolates were unavailable (collected before the harmonization of FNC and CIPARS farm surveillance methods) but full data were available from ON<sub>SS1b</sub> and BC<sub>SS2a</sub>. Percentage of resistance among *E. coli* and 95% CI were organized according to the Veterinary Drugs Directorate (VDD), Health Canada’s categorization of antimicrobials according to their importance in human medicine (22) (Figure 1). Resistance to ceftriaxone, a VDD Category I antimicrobial, was relatively low (<1% or 1 isolate in each sentinel site). Three isolates (5%) from ON<sub>SS1b</sub> and 4 isolates (4%) from BC<sub>SS2a</sub> were resistant to gentamicin. In BC<sub>SS2a</sub>, resistance to all other antimicrobials was relatively low (0% to 9%, depending on the antimicrobial) except for tetracycline at 26% (95% CI: 18% to 38%). In ON<sub>SS1b</sub>, 69% (95% CI: 42% to 82%) of *E. coli* isolates were resistant to tetracycline.

There were *E. coli* isolates that exhibited resistance to one or more of the antimicrobial drugs tested. In ON<sub>SS1b</sub>, there were 11 resistance patterns observed (including susceptible isolates) and the prevalence for each pattern ranged from 2% (5 various resistance patterns) to 37% (tetracycline) with a corresponding low percentage of susceptible isolates (27%) (Figure 2). In contrast, diverse resistance patterns (*n = 15*) were detected from BC<sub>SS2a</sub> isolates and the prevalence ranged from 1% (11 various patterns) to 16% (tetracycline) with a corresponding high percentage of susceptible isolates (69%) (Figure 3). In ON<sub>SS1b</sub>, 2% of isolates exhibited resistance to 7 and 8 antimicrobials (4 to 6 classes) and in BC<sub>SS2a</sub>, 2% of isolates exhibited resistance to 8 antimicrobial drugs (5 classes). All of the *Salmonella* isolates were susceptible to all the antimicrobials tested.

*Campylobacter* isolated from ON<sub>SS1a</sub> (*n = 4*) and ON<sub>SS1b</sub> (*n = 3*) exhibited resistance to ciprofloxacin, a fluoroquinolone class of antimicrobials belonging to VDD Category I. In both sentinel sites, *C. jejuni* ciprofloxacin resistant isolates originated from the same farm. In ON<sub>SS1a</sub>, 38% (95% CI: 16% to 66%) and in ON<sub>SS1b</sub>, 78% (95% CI: 42% to 94%) of *Campylobacter* isolates exhibited resistance to tetracycline (Figure 3). The 7 ciprofloxacin resistant isolates from Ontario (both sites) exhibited the ciprofloxacin-nalidixic acid-tetracycline resistance pattern.

Discussion

This study evaluated the prevalence of *E. coli*, *Salmonella*, and *Campylobacter* using 2 different sample matrices, serotype (i.e., *S. Enteritidis*), species (i.e., *Campylobacter* spp.), and resistance of isolates to the range of antimicrobials routinely tested by CIPARS. Our analysis indicates that *E. coli* and *Salmonella* can be isolated from either of the sample matrices, fecal samples, or environmental sponge swabs, using routine culture methodology. In the Ontario sites, the recovery of *Salmonella* varied depending on the surveillance year, but overall, *Salmonella* prevalence rates in our study were lower (8% and 29% in Ontario and 7% in British Columbia) compared to previous studies in layers conducted by CIPARS (42% of spent hens) (12) and in *Salmonella* barn and flock level prevalence, respectively) (13). These initial findings provided a descriptive landscape of current monitoring efforts, laboratory methods, and the potential contribution of chicken egg layers in the ecology of foodborne zoonotic bacterial pathogens in Canada.

With limited data collected so far by both CIPARS and FNC, temporal patterns of *Salmonella* prevalence could not be determined as the studies varied in terms of sampling design, age of the flock at sampling, geographical locations, study timeframe (i.e., sampling
Bacterial diseases continue to be diagnosed in laying hens in major categories of importance in human medicine, as outlined by the Veterinary Categories II to IV indicate categories of importance in human medicine, as outlined by the Veterinary FoodNet Canada sentinel sites. Roman numerals II to IV indicate categories of importance in human medicine, as outlined by the Veterinary FoodNet Canada sentinel sites.

Campylobacter in layer flocks from Canadian Integrated Program for Antimicrobial Resistance Surveillance and FoodNet Canada sentinel sites. Roman numerals II to IV indicate categories of importance in human medicine, as outlined by the Veterinary Drugs Directorate.

The overall intent of the SC-SC program is to improve the quality and microbiological safety of eggs and to reduce S. Enteritidis entering the food chain. The absence of egg-associated outbreaks in Canada in recent years indicates that this food safety program plus the intervention, which involves the eradication of positive flocks and a compensation program(b), and post-eradication measures (cleaning and disinfection) are working. However, in the context of a comprehensive and harmonized public health surveillance program, such as CIPARS and FNC, the ability of the current SC-SC methodology for detecting other enteric pathogens such as Campylobacter appears to be limited. Previous studies in layers that have used voided fecal/cecal samples found high Campylobacter recovery rates (24,25), which are directly reflective of bird-level Campylobacter compared to other sample matrices such as environmental swabs, which are likely more reflective of the barn or farm environmental flora. The low recovery rates from the sponge swabs could be due to the presence of environmental flora or the lack of transport media, thus requiring further validation of the technique. By contrast, although there was some variability, voided fecal/cecal samples performed consistently with the methods used by CIPARS and FNC in other poultry sectors such as broiler chickens and turkeys (9). Exploration of other farm-level methods similar to a study in the United States, which used a combination of environmental swabs taken from surfaces with visible fecal contamination including system wires, nest boxes, scratch pads, and fecal swabs plus shell pools (26–28), could be an option to enhance Campylobacter recovery. It is known that layer flocks could be colonized internally with Campylobacter (29) and regardless of the housing structure (cage and modifications of the cage systems), the organism could persist in the barn environment (26–28). As such, other than methodological validation of the sponge swab technique, the public health impact of layer flock exposures or egg consumption associated Campylobacter illnesses in people and the contamination pathways leading to human illness (i.e., dissemination via the food chain, via egg handling or environment) also warrant further research.

The antimicrobial resistance observed in E. coli was relatively infrequent compared to other poultry sectors. The moderate to high prevalence of resistance to certain antimicrobials (e.g., tetracycline and streptomycin), resistance to multiple drugs, and resistance to cephalosporins and fluoroquinolones, although comparatively lower than broiler chickens and turkeys, are somewhat unexpected. It is generally supposed that during the laying phase, exposure to antimicrobials in laying flocks is limited because of potential drug residues in table eggs (30); however, there are no published data to corroborate that assumption. In general, AMR prevalence and AMR profiles of isolates from Ontario differed from British Columbia. Bacterial diseases continue to be diagnosed in laying hens in major egg-producing provinces (10,11) including salpingitis/egg peritonitis and early systemic bacterial infections, but without animal health/operational farm-level information, it is impossible to assess the factors or drivers for AMR in layers. Surveillance timeframes and provincial variations in drug use could have played a role in the variations in AMR. The sample matrix used in the surveillance design could also have had an impact, as freshly voided fecal samples (or cecal samples) may be more reflective of a relatively recent AMU selection pressure. The CIPARS farm program (9) routinely uses fecal samples, as do many other food safety and AMR surveillance programs (31,32), because of the consistency in AMR profiles observed and the ability to detect changes in AMR prevalence over time. In contrast, the sponge swab, as previously described, may be reflective of the microbial flora within the farm/barn environment, where AMR dynamics distinct from the gut flora are potentially occurring. External to the bird’s immediate environment, exposure of the organisms to disinfectants and other cleaning agents and the presence of vectors could be playing a role.

The AMR findings warrant monitoring of AMU at all phases of egg production (e.g., hatchery, pullet, and laying phases) to understand the emergence of AMR in layer flocks in Canada. To the best of our knowledge, this is the first evidence that Campylobacter spp. and E. coli resistant to multiple antimicrobials are present in layer flocks in Canada. Although the environmental sponge swabs yielded comparable recovery rates for E. coli and comparable Salmonella prevalence rates, the use of fecal samples appears to be the most appropriate matrix for public health surveillance that includes Campylobacter and to inform a comprehensive foodborne pathogen intervention and AMU stewardship in the layer sector. In light of the changes in AMU regulations in Canada (33) and AMU reduction called for by the larger poultry industry group (34,35), the utility of
fetal samples could also be explored for animal health surveillance (e.g., *Clostridium perfringens*, *Brachyspira* spp., *Eimeria* spp.). This approach could allow across-species harmonization and methodological harmonization of farm level surveillance for layers, such as those used in the United States (14) and in the European Union Member States (19). However, the CIPARS and FNC programs are voluntary in nature and have limited flock coverage compared to the SC-SC, which is mandatory for all layer flocks; thus, opportunities for synergies could be explored to enhance the sustainability of these surveillance and monitoring programs.

In summary, inclusion of layer flocks in a surveillance program with a public health theme would contribute to the understanding of the ecology of AMR in the poultry industry and enable comprehensive source attribution and risk mitigation of foodborne illnesses and AMR using a One Health approach.

**Acknowledgments**

The authors sincerely acknowledge the egg producers and the provincial marketing boards for their participation and Dr. Rebecca Irwin and Lisa Landry for their guidance. CIPARS and FoodNet Canada are funded through the Public Health Agency of Canada.

**References**


Cholangiohepatitis is one of the most common pathological conditions affecting the liver of cats; second in prevalence to hepatic lipodosis (1,2). This disease condition is characterized by inflammation of bile ducts and surrounding parenchyma with infiltration of lymphocytes and plasma cells alone or in combination with neutrophils. Acute cholangiohepatitis in cats usually arises from ascending bacterial infections of the biliary tract and the inflammatory cells are mostly comprised of neutrophils (3,4). With time, occasionally, acute cholangiohepatitis progresses to a chronic non-suppurative phase, characterized by the infiltration of lymphocytes and plasma cells, prolation of bile ducts, and peri-portal fibrosis. At a later stage, cirrhosis develops that includes bridging fibrosis, nodular regeneration, and variable degrees of chronic inflammation (4). The clinical course of progression from the acute to chronic stage of this disease is always subtle and difficult to distinguish. The pathogenesis is not well-characterized in the literature, although immune-mediated mechanisms and chronic persistent bacterial infections have been discussed in conjunction with this condition (1,4). Although acute cholangiohepatitis can be effectively controlled by prompt identification and treatment of the inciting cause (3); the chronic form of
the disease has a guarded prognosis because the etiology is often unidentified (1,2,4).

Recently, in human medicine, there has been more emphasis on enhancing liver defense mechanisms as a treatment strategy for chronic liver disease, instead of targeting the initial inciting cause (5). Metallothionein (MT), an inducible intracellular heavy metal binding protein, has been proven to have hepatoprotective properties. The putative functions of MT include homeostasis, detoxification of heavy metals, and protection against oxidative stress by scavenging a wide range of oxidants and electrophiles (6–8). Metallothionein is capable of binding toxic metals such as cadmium, mercury, platinum, and silver, protecting cells and tissues from heavy metal-induced toxicity. The protein also acts as a major reserve for biologically essential metals such as zinc and copper (9). The anti-inflammatory, antifibrotic, and hepatocellular regenerative properties of MT have been explicated in previous studies in mice (5,10,11). In dogs, MT expression was found to be positively correlated with hepatocellular regeneration and negatively correlated to hepatic fibrosis (12). However, in a recent study in horses, no significant correlation was found between MT expression and Ki-67 expression with inflammation, fibrosis, and bile duct proliferation (13). These results suggest the possibility for species difference in MT expression, in relation to the histologic parameters in chronic liver inflammation.

An investigation of MT expression in the liver of cats affected by chronic cholangiohepatitis (CCH) has not been conducted previously.

### Table I. Demographic data for cats selected in this study from which liver samples originated. Samples were assessed for metallothionein and Ki-67 labelling, inflammation, fibrosis, and bile duct proliferation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Breed</th>
<th>Number of cats</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCH</td>
<td>Female</td>
<td>1 to 5</td>
<td>Balinese</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DLH</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DSH</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Siamese</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 to 10</td>
<td>DMH</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DSH</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown breed</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11 to 15</td>
<td>DSH</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Siamese</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown breed</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 to 20</td>
<td>Unknown breed</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>1 to 5</td>
<td>DSH</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown breed</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>Female</td>
<td>1 to 5</td>
<td>DSH</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown age</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>1 to 5</td>
<td>DMH</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown age</td>
<td>1</td>
</tr>
</tbody>
</table>

DLH — Domestic long hair; DMH — Domestic medium hair; DSH — Domestic short hair.
Table II. Data summary of 34 diseased and 5 normal liver samples assessed for metallothionein and Ki-67 labelling, inflammation, fibrosis, and bile duct proliferation.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Inflammatory cells</th>
<th>Fibrosis, 0–3</th>
<th>BDP</th>
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<th>MT labelling intensity, 0–3</th>
<th>Ki-67 positive hepatocytes, 0–3</th>
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MT — metallothionein; BDP — bile duct proliferation.

a Case number: 1–34, liver samples with chronic cholangiohepatitis; 35–39, normal liver samples.
b Mean number of inflammatory cells surrounding 5 random portal areas.
c Mean fibrosis score by 2 independent pathologists in 5 random HPF (40×).
d Mean number of bile ducts counted in 5 random portal areas by 2 independent pathologists.
e Mean number of hepatocytes with positive MT labelling in 10 random HPF (40×).
f Mean MT labelling intensity in 10 random HPF (40×).
g Mean number of hepatocytes with positive Ki-67 staining in 10 random HPF (40×).
The pathogenesis of the disease is poorly understood and the etiology is often not identified (2). Therefore, cats, similar to humans, may benefit from treatment modalities enhancing the expression of MT when CCH is diagnosed.

The objective of this study was to evaluate the expression of MT in formalin-fixed paraffin-embedded liver sections from cats affected by CCH using immunohistochemistry and to assess the correlation between its expression and hepatic regeneration, inflammation, fibrosis, and bile duct proliferation (BDP). It was hypothesized that MT expression is positively correlated with hepatic regeneration and inflammation and negatively correlated with BDP and fibrosis.

**Case selection and histologic scoring**

Case records from 2009 to 2018, inclusive, from the archives of Prairie Diagnostic Services Inc., Saskatoon, Saskatchewan, Canada, were searched for cases with the histologic diagnosis of CCH. Hematoxylin and eosin (H&E) stained glass slides, corresponding to these cases were reviewed by a Board-certified pathologist (A.N.A) to confirm the initial diagnosis and 34 cases (Tables I, II) were selected if at least 1 cm² of liver tissue was available for examination. Liver tissues from these selected cases were scored for inflammation, fibrosis and BDP, which were the diagnostic criteria for CCH as outlined by the World Small Animal Veterinary Association Liver Standardization Group (14).

High resolution microscopic images were captured using an Olympus DP70 digital camera and an Olympus microscope at 40× magnification to score inflammation. The number of neutrophils, lymphocytes, and plasma cells infiltrating the hepatic portal tracts were counted in 5 random high-power fields (40×) using Image Pro 9.2 software (Media Cybernetics, Rockville, Maryland, USA), by individually tagging the inflammatory cells; a mean was then calculated. For fibrosis scoring, all slides were stained with Masson’s Trichrome and scored 0, 1, 2, or 3, where 0 indicated the absence of fibrosis; 1 indicated presence of fibrosis around portal tracts only; 2 represented the presence of nonbridging fibrosis extending beyond portal or centrilobular areas into the hepatic parenchyma; and 3 was associated with the presence of bridging fibrosis. Bile duct proliferation was assessed by counting the number of bile ducts in 5 random high-power fields (40×) locations that contained at least 1 bile duct and a mean was calculated. Fibrosis and BDP were independently assessed by 2 experienced pathologists (A.N.A and A.L.A) and the means of their scores were taken.

**Immunohistochemistry and scoring for MT and Ki-67 expression**

The slides for MT and Ki-67 expression were stained using a slightly modified version of a staining protocol previously adopted by Verhoef et al (13). Briefly, consecutive 4-μm sections from formalin-fixed, paraffin-embedded tissue blocks were mounted on ProbeOn Plus (Fischer Scientific, Waltham, Massachusetts, USA) charged glass slides. The slides were oven-baked at 60°C for 1 h, then deparaffinized in xylene for 5 min, followed by rehydration in graded concentrations of alcohol (100%, 95%, and 70%) for 5 min each. The slides were then rinsed in distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min followed by a 5-minute rinse in phosphate-buffered saline with Tween 20 (PBST). The slides were incubated for 20 min in Tris ethylenediamine tetra-acetic acid (EDTA) buffer (pH 9) in a Dako PT Link water bath set at 97°C.

The primary antibodies for MT (clone E9, mouse anti-horse, monoclonal IgG1; Dako, Burlington, Ontario) and Ki-67 (clone MIB-1, mouse anti-human, monoclonal IgG1; Dako) were diluted to 1:1000 and 1:50, respectively, using antibody diluent (Dako) and incubated at room temperature for 30 min followed by a 5-minute rinse in PBST. Color development was performed using diaminobenzidine buffer (DAB) and DAB chromogen buffer. The slides were counterstained with haematoxylin for 4 min and mounted with cover slips. Antibody specificity was assessed both by exclusion of primary antibody (omission) and substitution with an irrelevant antibody. Histologically normal cat kidney and lymph node tissues were used as positive controls for MT and Ki-67, respectively. Five histologically normal liver tissues were also stained with MT and Ki-67, for comparison.

Hepatocellular MT expression was evaluated by the following 2 methods: i) The number of hepatocytes with positive MT staining in 10 random high-power fields (40×) was counted using an intraocular square grid measuring 0.25 mm × 0.25 mm and a mean was calculated; and ii) MT labelling intensity was scored subjectively from 0 to 3 in 10 random high-power fields (40×) and a mean was calculated. A score of 0 indicated no labelling; 1 indicated the presence of weak labelling intensity; 2 represented the presence of moderate labelling intensity; and 3 was associated with the presence of strong labelling intensity. Liver regeneration was assessed by counting the number of hepatocytes with positive Ki-67 staining (Ki-67 expression) in 10 random high-power fields (40×) using the same grid and a mean was calculated.

Figure 1. A — Photomicrograph of a liver from a cat affected by chronic cholangiohepatitis (10×). Note the centrilobular zones (arrowhead) devoid of inflammatory cells and the portal zones surrounded by abundant infiltration of lymphocytes and plasma cells, together with fibrosis and bile duct proliferation (arrows). B — Portal zones contain multiple bile ducts surrounded by abundant infiltration of lymphocytes and plasma cells. Case no. 2. H & E staining.
Statistical analysis

Statistical data were analyzed using SAS 9.4 (SAS Institute, Cary, North Carolina, USA). Correlation between MT expression and Ki-67 expression, hepatocellular inflammation, fibrosis, and BDP were analyzed using Spearman’s rank correlation test. Inter-observer variations in fibrosis and BDP scoring were also assessed using Spearman’s rank correlation test. Differences were considered statistically significant when \( P < 0.05 \).

Results

Numerical values for all the evaluated parameters are presented in Table II. A variable degree of inflammation was present in all liver sections affected by CCH with lymphocytes, plasma cells, and neutrophils being primarily seen (Figure 1). Masson’s trichrome staining highlighted increased collagen deposits (blue) with increased severity of fibrosis (Figures 2A–C).

Metallothionein expression was present in 94.1% of cases (32/34). The expression was mostly cytoplasmic within hepatocytes, which rarely exhibited variable nuclear staining (Figure 3). The mean number of hepatocytes with positive MT staining ranged from 0 to 89 cells in 10 random high-power fields (40×). Mean MT labelling intensity ranged from 0.6 to 2.9 in 10 random high power (40×) fields (Tables II, III; Figures 4, 5).

Positive Ki-67 expression was present in both hepatocytes and Kupffer cells in 35% of the liver samples (Figure 6). Ki-67 expression was absent in 15% of samples and present only in Kupffer cells in 50% of samples. In liver samples with positive Ki-67 expression, the mean number of positive hepatocytes ranged from 0 to 2.8 in 0.625 mm\(^2\) (Figure 7; Table III).

In 37% of cases (16/43) MT labelling intensity was noted to be pronounced towards the centrilobular zone and was very weak or absent towards the portal zone (Figure 7A, B). A significant correlation (Table IV) was observed between the number of hepatocytes with positive MT staining and inflammation \((r = 0.36, P = 0.03)\) and also between MT labelling intensity and inflammation \((r = 0.37, P = 0.03)\). No correlation was found between the number of hepatocytes with positive MT staining, MT labelling intensity, or Ki-67 expression and fibrosis or BDP.
Table III. Mean, standard deviation, minimum and maximum values of variables assessed in 34 liver samples with histologic diagnosis of cholangiohepatitis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
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<td>30.1</td>
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<td>89.3</td>
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<td>0.5</td>
<td>0.6</td>
<td>2.9</td>
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<td>0.6</td>
<td>0</td>
<td>2.8</td>
</tr>
<tr>
<td>Inflammation</td>
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<td>193</td>
<td>6.6</td>
<td>999</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>2.1</td>
<td>0.6</td>
<td>0.5</td>
<td>3</td>
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<td>BDP</td>
<td>4.5</td>
<td>3.1</td>
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<td>12.4</td>
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SD — Standard deviation; MT — Metallothionein; BDP — Bile duct proliferation.

Assessment of interobserver variation revealed significant correlation among the histologic scores of the 2 pathologists for BDP ($r = 0.73, P < 0.01$) and fibrosis ($r = 0.53, P < 0.01$). MT and Ki-67 expression in hepatocytes in normal liver samples were very weak or absent.

Discussion

Metallothionein is a family of small proteins, which play an essential role in metal homeostasis, inflammation, and regeneration. Four major isoforms of MT have been identified (MT-I — IV) (7,9,13). MT-I and MT-II are closely related, and considered ubiquitous in mammalian cells and are induced by stress (7,9,15). MT-III is mainly expressed in the central nervous system where it is thought to inhibit neuronal growth. The levels of MT-III have been found reduced in the brain of patients affected by Alzheimer’s disease (16). MT-IV expression is restricted to squamous epithelium where it plays a role in zinc and copper homeostasis (17).

Studies in MT-knockout and wild type mice have demonstrated that the induction of MT is critical at times of acute stress (18). In the liver, MT gene expression is rapidly upregulated by various stressors including hot and cold environmental conditions, heat burn, strenuous exercise, and endotoxin-induced systemic inflammation resulting from bacterial infections. Metallothionein is, therefore, considered an acute phase protein (19). The role of MT in inflammation is imparted by its ability to scavenge a wide range of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide (7). Metallothionein also acts as a zinc chaperone for the activation of matrix metalloproteiaines (MMPs) (15), which are zinc-dependent enzymes, involved in degradation of collagen and extracellular matrix components, thus promoting tissue repair and regeneration during chronic inflammation. MMP’s are also regulators of cytokines and chemokines and thus thought to play a role in inflammation and immunity (20).

The objective of our study was to examine the expression of MT in feline liver tissues affected by CCH and to correlate its expression with liver regeneration, inflammation, fibrosis, and BDP. “MT expression” will be used hereafter to refer to both the number of hepatocytes with positive MT staining and MT labelling intensity as they are discussed together. A significant positive correlation between MT expression and inflammation was observed, which supports the idea that MT plays a role in inflammation in...
CCH in cats. A similar positive correlation between MT expression and inflammation has been documented previously by Sridharan et al (12), in a study conducted in dogs affected by chronic liver disease. Previous studies in mice have investigated the protective role of MT in liver inflammation and toxicity conditions (7,8,21). Increased pulmonary MT expression was observed following chemical induced oxidative stress, with cytoprotective effects (22). Increased hepatic MT concentrations are reported to occur during the early phase of inflammation in bacterial infections and endotoxemia (23). A few studies utilized wild type and MT knockout mice to demonstrate the regulatory effect of MT in inflammation and toxicity conditions. Histopathological and ultrastructural examination of lung tissue following intratracheal administration of lipopolysaccharide, demonstrated significant increase in pulmonary edema and inflammation in wild type mice compared to MT knockout mice (8). In another such study, wild type mice demonstrated a rise in MT protein concentrations, with significantly lower levels of oxidative damage (lipid peroxidation) and tissue injury (necrosis), when compared to knockout mice, following induction of acute hepatotoxicity by intraperitoneal injection of thioacetamide (21). Studies have shown that MT knockout mice have an increased sensitivity to harmful metals such as cadmium, mercury, and arsenic, oxidative stress, chemical carcinogenesis, and neurodegenerative diseases compared to wild type mice, which supports the idea that MT plays an essential role in heavy metal-induced toxicity (24). In human patients with chronic hepatitis C, MT expressed in hepatocytes is shown to play a defensive role in oxidative stress by limiting inflammation and viral replication (11). Our findings are in line with the previous research investigating the role MT in inflammatory conditions (12,25) and likely represents a protective response of MT to the increased oxidative stress occurring during inflammation. Being an acute phase protein upregulated during acute inflammation, the finding of a correlation between MT expression and chronic inflammation in our study may indicate that tissue injury is still occurring.

A gradual increase in the MT labelling intensity towards the centrilobular areas was noticed in 47% of tissue samples, while inflammation was concentrated around the portal areas. In the remaining 53% samples, MT labelling intensity was homogeneous, without a zonal predilection. Yin et al (25), reported that immune cells migrate chemotactically in the presence of a gradient of MT.

**Table IV. Spearman rank correlation analysis of MT, Ki-67 expression and selected histologic parameters in 34 liver samples with histologic diagnosis of cholangiohepatitis.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>MT-positive cells in 0.625 mm² P-value</th>
<th>r</th>
<th>MT labelling intensity P-value</th>
<th>r</th>
<th>Ki-67 positive cells 0.625 mm² P-value</th>
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<td>0.366</td>
<td>0.03*</td>
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MT — Metallothionein; BDP — Bile duct proliferation.

* Mean number of inflammatory cells surrounding 5 random portal areas.

**Figure 7. Photomicrograph of a liver from a cat affected by chronic cholangiohepatitis, immunohistochemically stained for MT expression (10×).** Note the labelling intensity is strongest in cells near the centrilobular zone and weak in cells near the portal zone. A — Case no. 9. B — Case no. 11. DAB staining & hematoxylin counterstaining.
Nagamine and Nakajima (11) observed that MT proteins were mostly observed around portal tracts together with inflammation and fibrosis in liver biopsies from human patients with chronic hepatitis C virus infection. The increased MT labelling intensity in hepatocytes close to the centrilobular zones observed in less than half of the samples in our study seems intriguing, considering the fact that the hepatocytes adjacent to the site of inflammation are more prone to oxidative stress compared to those away from these sites. A higher MT expression in those cells close to inflammation would be expected, which is just the opposite of what we found. In previous studies investigating MT expression in chronic liver disease of dogs (12) and horses (13), no zonal variation in MT labelling intensity was reported. It is plausible that there may be factors, yet unknown to us, that may influence MT expression in cats, and further studies may be necessary to unravel the mechanism behind this.

No significant correlation was observed between MT expression and other parameters associated with chronic inflammatory conditions, such as liver fibrosis and BDP. Sridharan et al (12) reported a significant negative correlation between the percentage of hepatocytes with positive MT staining and liver fibrosis in dogs. These results were in concordance with previous studies conducted in mice. Wild type mice exposed to long-term treatment of carbon tetrachloride developed irreversible liver fibrosis. Fibrosis was reversed when MT was given intravenously through adenoviral gene delivery (10). These results provide evidence that MT plays a role against fibrosis. Similar to our findings, Verhoef et al (13) did not find any significant correlation between liver fibrosis and MT expression in horses. Metallothionein expression was not related to BDP, which is consistent with the findings of Sridharan et al (12) and Verhoef et al (13), who also failed to observe a correlation between MT expression and BDP in studies conducted in dogs and horses, respectively. Overall, the absence of correlation between MT expression and liver fibrosis or BDP in our study may suggest that MT expression is not directly related to liver fibrosis or BDP in cats. It could be speculated that different species vary in their MT expression in relation to these parameters, as previously proposed by Verhoef et al (13). The absence of a correlation could also be related to the small sample size used in our study.

Previous reports have shown that MT plays an essential role in hepatic regeneration (26,27). Following liver injury, MT production is induced to meet the increased demands of zinc in regenerating cells (26). In a study comparing hepatic regeneration following partial hepatectomy in wild type and MT knockout mice, wild type mice demonstrated a significant hepatic regeneration compared to MT knockout mice (27). Following exposure to the hepatotoxin, thiocetamide, MT knockout mice showed a significant reduction in their potential for hepatic regeneration (21). A significant but weak correlation between Ki-67 and MT expression was also noticed in dogs by Sridharan et al (12). However, we did not observe a significant correlation between MT expression and Ki-67 expression in our study. Similar observations were made by Verhoef et al (13) in horses. The authors also identified a positive correlation between Ki-67 expression in Kupffer cells and MT expression in hepatocytes of equine livers with chronic hepatitis, but such a correlation was not present in our samples. Hepatocytes with positive Ki-67 expression in our samples ranged from 0 to 2.8. This range is much lower than the values obtained by Sridharan et al (12) in dogs, but comparable with the values of the horse study by Verhoef et al (13). The low Ki-67 expression in the liver of cats with CCH may indicate a low regenerative capacity in these livers and may explain the guarded prognosis in cats affected by this disease.

A few disadvantages in our approach was the small sample size and use of a single marker (Ki-67) to assess hepatic regeneration. However, this is the first study that examined MT expression in the liver of cats and future studies may consider experiments with large cohorts incorporating age, sex, and breed of animals in the study design, to identify if any of these factors can influence MT expression and liver regeneration in cats affected with CCH. The use of multiple markers (28) instead of a single marker (Ki-67) could also be attempted, for a better estimation of regenerating hepatocytes. Inflammation is pivotal in the body’s defense against infectious agents; however, at high levels, inflammation-induced oxidative stress, can cause deleterious effects to cell membranes, nucleic acids, and proteins, and may lead to tissue necrosis and loss of function. Metallothioneins are considered part of the innate and adaptive immune defense mechanism of the body, and thought to have protective effects against inflammation-induced tissue injury (18). Apart from being primarily an intracellular protein, MT is also present in extracellular compartments such as liver sinusoids, serum, urine, and bronchoalveolar spaces. Immunomodulatory effects of extracellular MT are demonstrated by its ability to direct chemotaxis of leucocytes (25). The beneficial effects of MT as an anti-inflammatory molecule and its role in tissue repair and regeneration indicate that MT plays a protective role during acute and chronic inflammation. The positive correlation between MT expression and inflammation in CCH in cats in our study substantiates this argument and suggests that chronic inflammatory conditions can augment the production of this protein in the liver to improve host defense. It may be feasible, therefore, to explore the possibility of using MT as a therapeutic agent for the manipulation of inflammation in vivo.

**Acknowledgments**

We thank Dr. Sarah Parker for assistance with the statistical analysis; Melissa Koehnlein and Jolanda Verhoef for assistance with immunohistochemistry. This study was funded by a grant from the Western College of Veterinary Medicine Companion Animal Health Fund, Saskatoon, Saskatchewan, Canada.

**References**

Changes in serum protein electrophoresis profiles and acute phase proteins in calves with diarrhea

Kyoung-Seong Choi, Jin-Hee Kang, Hyung-Chul Cho, Do-Hyeon Yu, Jinho Park

Abstract

Calf diarrhea leads to substantial economic losses in the livestock industry worldwide due to medical treatment costs, retarded growth performance, and even death. The objective of this study was to investigate changes in serum protein profiles and acute phase proteins in calves with diarrhea and identify the association between these changes and diarrhea. A total of 185 Korean beef calves were used and divided into 3 groups by age: 1 to 10 days ($n=46$), 11 to 20 days ($n=65$), and 21 to 30 days ($n=74$). Blood and fecal samples were collected from each calf. Serum concentrations of total protein, protein fractions (albumin, $\alpha$-globulin, $\alpha2$-globulin, $\beta$-globulin, and $\gamma$-globulin), haptoglobin (Hp), and serum amyloid A (SAA) were analyzed. Compared to calves without diarrhea, calves with diarrhea had significantly lower albumin concentrations at 11 to 20 days and 21 to 30 days of age ($P=0.017$ and $P=0.000$, respectively) and significantly higher $\alpha1$-globulin fractions at 21 to 30 days of age ($P=0.01$). Interestingly, $\alpha2$-globulin fractions were significantly higher in diarrheic calves in all age groups, whereas $\gamma$-globulin fractions were significantly lower in calves with diarrhea aged 1 to 10 days, compared with normal animals. In calves with diarrhea, the concentration of Hp was significantly higher, whereas SAA levels were not different between normal and diarrheic calves. In addition, a positive correlation was found between $\alpha2$-globulin and Hp ($P=0.0004$). Taken together, these results provide useful information about the use of serum protein profiles and Hp as prognostic and diagnostic markers for animal health status.

Résumé

La diarrhée des veaux entraîne des pertes économiques substantielles dans l’industrie de l’élevage dans le monde entier en raison des coûts des traitements médicaux, du retard de croissance et même de la mort. L’objectif de cette étude était d’étudier les changements dans les profils des protéines sériques et les protéines de la phase aigüe chez les veaux souffrant de diarrhée et d’identifier l’association entre ces changements et la diarrhée. Un total de 185 veaux de boucherie coréens ont été utilisés et répartis en trois groupes par âge : 1 à 10 jours ($n=46$), 11 à 20 jours ($n=65$) et 21 à 30 jours ($n=74$). Des échantillons de sang et de matières fécales ont été prélevés sur chaque veau. Les concentrations sériques de protéines totales, les fractions protéiques (albumine, $\alpha1$-globuline, $\alpha2$-globuline, $\beta$-globuline et $\gamma$-globuline), d’haptoglobine (Hp) et d’amylodé sérique A (SAA) ont été analysées. Par rapport aux veaux sans diarrhée, les veaux souffrant de diarrhée avaient des concentrations d’albumine significativement plus faibles à 11 à 20 jours et 21 à 30 jours d’âge ($P=0.017$ et $P=0.000$, respectivement) et des fractions d’$\alpha1$-globuline significativement plus élevées à 21 à 30 jours d’âge ($P=0.01$). Il est intéressant de noter que les fractions d’$\alpha2$-globuline étaient significativement plus élevées chez les veaux diarrhéiques de tous les groupes d’âge, tandis que les fractions de $\gamma$-globuline étaient significativement plus faibles chez les veaux souffrant de diarrhée âgés de 1 à 10 jours, par rapport aux animaux témoins. Chez les veaux souffrant de diarrhée, la concentration de Hp était significativement plus élevée, tandis que les niveaux de SAA n’étaient pas différents entre les veaux normaux et diarrhéiques. De plus, une corrélation positive a été trouvée entre l’$\alpha2$-globuline et Hp ($P=0.0004$). Pris ensemble, ces résultats fournissent des informations utiles sur l’utilisation des profils de protéines sériques et de Hp comme marqueurs pronostiques et diagnostiques de l’état de santé des animaux.

(Traduit par Docteur Serge Messier)

Introduction

Diseases that occur during breeding of cattle can lead to substantial economic losses in the livestock industry due to medical treatment costs, retarded growth performance, and even death. Neonatal calves have a greater tendency to contract various diseases from birth, which in serious cases, may result in mortality (1,2). In particular, exposure to infectious agents through the oral route and the respiratory tract contributes to disease occurrence in calves, as newborn calves are easily exposed to external environmental contaminants during delivery. Therefore, delivery facilities housing neonatal calves should always be kept clean and isolated from adult cattle to prevent potential infections from pathogens. Overall, prevention and control of diseases during this period are very important.
because if these factors are neglected, poor animal management systems and inappropriate breeding methods can lead to increased disease incidence.

Neonatal calf diarrhea is associated with a high worldwide mortality rate within the first month of life (3). Calf diarrhea may be attributed to multifactorial etiologies, including various infectious agents, animal management systems, hygiene, and nutrition (4). Of the numerous infectious agents causing calf diarrhea, the focus is mainly on individual pathogens such as bovine viral diarrhea virus (BVDV), coronavirus, norovirus, rotavirus, Clostridium spp., Escherichia coli, Salmonella spp., Cryptosporidium parvum, Eimeria spp., and Giardia duodenalis (5–9). In addition, because neonatal calves are highly vulnerable to these pathogens, management of animal facilities and care, appropriate feeding, and immunity status of calves during this period are very important in preventing major diseases such as diarrhea and respiratory disorders. Therefore, immediate and rapid colostrum intake after calving should be performed, as it is necessary for preventing diseases in neonatal calves that are completely dependent on passive immunity.

Acute phase proteins (APPs) are plasma proteins synthesized by hepatocytes that appear during the acute phase response, including infection, tissue injury, neoplasia, and inflammation (10). Acute phase proteins serve as the core of the innate immune response and are conserved across animal species (11,12). Changes in the concentrations of APPs are recognized as a useful tool for evaluating cattle health, and haptoglobin (Hp), serum amyloid A (SAA), fibrinogen, and α1-acid glycoprotein are the most common APPs in cattle (8,13). Several studies have shown that APPs have good properties as markers of respiratory infections in calves (14–17); however, there are limited data available on APPs as disease markers for calf diarrhea.

Immunoglobulin concentrations in serum can be evaluated through electrophoretic techniques. The determination of serum protein fractionation is of important diagnostic value in clinical biochemistry and serum protein electrophoresis is a laboratory test that separates serum proteins into albumin and globulin. The latter is then subdivided into α-, β-, and γ-globulins (18–21). Therefore, the objective of this study was to investigate the differences in serum protein profiles and APPs between healthy and diarrheic calves during the first month after birth and to identify any association between the changes in serum protein patterns and/or APPs and diarrhea. The results obtained in this study may provide valuable information for veterinary clinicians to predict and treat animal physiological status.

Materials and methods

Experimental animals

This study was conducted on 185 Korean beef calves (≤ 30 d of age) being raised in the Republic of Korea. Calves were classified by age into 3 groups: 1 to 10 d (n = 46), 11 to 20 d (n = 65), and 21 to 30 d (n = 74) (Table I). The physical activity of all calves used in this experiment was noted as “bright,” which is a state of normal response to stimuli. No dehydration was observed in diarrheic calves.

Table I. Classification and total numbers of calves used in this study.

<table>
<thead>
<tr>
<th>Classification of calves</th>
<th>1 to 10 d</th>
<th>11 to 20 d</th>
<th>21 to 30 d</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>39</td>
<td>48</td>
<td>105</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>28</td>
<td>26</td>
<td>26</td>
<td>80</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>65</td>
<td>74</td>
<td>185</td>
</tr>
</tbody>
</table>

Blood and fecal sampling, and detection of diarrheal pathogens

Blood was collected from the jugular vein of each calf. Samples were divided into tubes and transferred to the laboratory on ice for a complete blood (cell) count (CBC) and a serum test. An experienced veterinarian directly collected feces from the rectum and fecal samples were transported to the laboratory. Feces were subdivided into solid, semi-solid, loose, and watery stools. Feces that were considered solid and semi-solid were classified as normal and those that were loose and watery were classified as diarrhea. DNA and RNA were extracted from 200 mg of each fecal sample using an AllPrep PowerFecal DNA/RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s directions and stored at −20°C until ready for use. Real-time polymerase chain reaction (RT-PCR) was performed to detect BVDV, coronavirus, rotavirus, E. coli and Salmonella spp., C. parvum, and G. duodenalis in the samples as previously described (22,23). For the detection of Eimeria species, fecal samples were analyzed for the presence of oocysts using the flotation method with Sheather’s solution (saturated sugar solution; specific gravity = 1.28) and examined microscopically (400× magnification). One or more of the pathogens related to diarrhea were detected in diarrheic calves, whereas no pathogens under investigation were detected in normal calves.

Serum protein gel electrophoresis

Serum samples were separated from blood by centrifugation. Subsequently, agarose gel electrophoresis was performed to analyze 5 protein fractions (albumin, α1-globulin, α2-globulin, β-globulin, and γ-globulin) using a semi-automated agarose gel electrophoresis system (HYDRASYS 2; Sebia, Camberley, United Kingdom), following the manufacturer’s protocols. Briefly, 30 μL of serum was subjected to the microtechnique assay, electrophoresed for 35 min, stained for 5 min, de-stained for 5 min, and cleared for 30 s. Excess solution was removed with a glass rod and samples were dried for 10 min and then measured by optical density scanning (HYDRASYS; Sebia). Normal serum was used as a control for measurement accuracy. The results of the serum protein electrophoresis gel were reviewed and interpreted by a laboratory expert.

Acute phase proteins

Analyses for Hp and SAA were performed in serum. The concentrations of Hp were assessed using commercial colorimetric kits (Tridelta Development, Kildare, Ireland) based on the hemoglobin-binding assay. The SAA was analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) kits (Tridelta Development). The optical densities were read on a microplate reader (BioTek Instruments,
Table II. Concentrations of total protein and albumin and globulin fractions (\(\alpha_1\), \(\alpha_2\), \(\beta\), and \(\gamma\)) in serum of calves according to age. Data are expressed as mean ± standard deviation. \(P\)-values were obtained using the Wilcoxon rank-sum test to compare normal calves with diarrheic calves.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Fecal consistency</th>
<th>1 to 10 d</th>
<th>11 to 20 d</th>
<th>21 to 30 d</th>
<th>1 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>Normal</td>
<td>6.6 ± 1.2</td>
<td>6.7 ± 0.7</td>
<td>6.1 ± 0.6</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>6.1 ± 1.5</td>
<td>6.3 ± 0.9</td>
<td>6.4 ± 0.9</td>
<td>6.3 ± 1.1</td>
</tr>
<tr>
<td>Albumin</td>
<td>Normal</td>
<td>2.8 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>3.7 ± 0.3</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>2.7 ± 0.7</td>
<td>3.0 ± 0.5**</td>
<td>3.2 ± 0.5***</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>(\alpha_1)-globulin</td>
<td>Normal</td>
<td>1.1 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td>0.8 ± 0.3***</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>(\alpha_2)-globulin</td>
<td>Normal</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>0.9 ± 1.4**</td>
<td>0.9 ± 1.0*</td>
<td>0.7 ± 0.3***</td>
<td>0.8 ± 1.0</td>
</tr>
<tr>
<td>(\beta)-globulin</td>
<td>Normal</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>1.1 ± 0.3***</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>(\gamma)-globulin</td>
<td>Normal</td>
<td>1.4 ± 0.9</td>
<td>0.8 ± 0.5</td>
<td>0.7 ± 0.4</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>0.6 ± 0.5**</td>
<td>0.8 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>

* \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\).

Winooski, Vermont, USA) at 630 nm for Hp and at 450 nm and 630 nm as a reference for SAA.

**Statistical analysis**

Data are presented as mean ± standard deviation (SD). Statistical analysis (i.e., Wilcoxon signed-rank test) of protein fractions between healthy and diarrheic calves was performed using SPSS Statistics version 25 for Windows (IBM, Armonk, New York, USA). A \(P < 0.05\) was considered significant. Correlations between Hp and a globulin selected from the diarrhea group were evaluated using Spearman’s correlation. Correlation coefficients \((r)\) < -0.4 and > 0.4 were considered to be significant negative and positive correlations, respectively.

**Results**

Of the pathogens examined from 80 diarrheic calves, rotavirus, BVDV, *E. coli*, *Eimeria* spp., *C. parvum*, and coronavirus were detected in 36 (45%), 13 (16%), 13 (16%), 11 (14%), 4 (5%), and 3 (4%) calves, respectively. *Giardia duodenalis* and *Salmonella* spp. were not found in diarrheic calves. The prevalence of rotavirus was the highest, whereas the incidence of coronavirus was the lowest in pre-weaned Korean native calves.

Overall, the concentrations of total protein increased with age in calves with diarrhea, but there were no significant differences noted (Table II). Albumin concentrations steadily increased with age in both normal and diarrheic calves and this increase was much greater in normal calves than in diarrheic calves (Table II). Albumin concentrations in calves with diarrhea were significantly lower at 11 to 20 d \((P = 0.017)\) and 21 to 30 d \((P = 0.000)\) compared with normal calves at those ages. The globulin fractions between normal and diarrheic calves are shown in Table I. The \(\alpha_1\)-globulin fractions were slightly higher in calves with diarrhea after 10 d than in normal calves, although statistical significance was observed only in diarrheic calves aged 21 to 30 d \((P = 0.001)\); Table II). The \(\alpha_2\)-globulin fractions were also significantly higher in calves with diarrhea compared with normal calves for all age groups (Table II). The \(\beta\)-globulin fractions decreased with age in normal calves, whereas \(\beta\)-globulin fractions gradually increased with age in calves with diarrhea and were significantly higher in calves with diarrhea aged 21 to 30 d \((P = 0.000);\) Table II). When comparing \(\gamma\)-globulin fractions between the 2 groups, the \(\gamma\)-globulin fractions in normal calves gradually reduced with age, whereas there was not much difference across ages for diarrheic calves. The \(\gamma\)-globulin fractions were significantly lower in calves with diarrhea aged 1 to 10 d \((P = 0.000);\) Table II).

The globulin composition ratio between normal and diarrheic calves is presented in Figure 1. The \(\alpha_1\)-globulin fractions were not different between the 2 groups, whereas the \(\alpha_2\)-globulin fractions were significantly higher in diarrheic calves of all ages compared to normal calves. The \(\beta\)-globulin fractions were significantly lower only in diarrheic calves aged 11 to 20 d. When compared to normal calves, the \(\gamma\)-globulin fractions in diarrheic calves were lower for all age groups and significantly lower in calves aged 1 to 10 d.

The concentrations of Hp and SAA were compared between normal and diarrheic calves. As shown in Figure 2, the Hp concentrations in calves with diarrhea of all age groups were significantly higher than in normal animals. Haptoglobin concentrations in the serum were at least 5-fold higher in calves with diarrhea compared to clinically healthy calves. The concentrations of SAA were higher in calves with diarrhea compared to normal calves, but differences were not statistically significant. A positive correlation was found between \(\alpha_2\)-globulin and Hp in calves with diarrhea \((P = 0.0004);\) Figure 3), whereas there were no correlations between \(\alpha_1\)-globulin and SAA in these calves.
This study used electrophoresis analysis to evaluate changes in serum protein and APP profiles due to diarrhea. The results showed that the composition of albumin and globulin fractions were markedly different between normal calves and calves with diarrhea. In terms of the globulin composition, the most significant differences between the 2 groups were observed in α2-globulin and γ-globulin fractions. The concentrations of Hp in the serum were significantly elevated in calves with diarrhea and this increase was associated with α2-globulin. These findings suggest that the changes in serum proteins (especially α2-globulin and γ-globulin) and Hp may be used as prognostic markers to predict and diagnose the health status of animals.

Albumin is the major protein component of serum. It is mainly produced in the liver and is involved in the transport of water or metabolites from tissues. One of the main functions of albumin is to maintain colloid osmotic pressure (24–27). Several studies have shown that albumin is decreased in cases of malnutrition, infection, chronic liver disease, and kidney disease. According to our results, albumin was decreased in calves with diarrhea compared to normal calves, indicating that diarrhea causes a loss of albumin in the contents of the intestine. In addition, the loss of albumin in calves with diarrhea increases when large volumes of fluid, ingesta, and hypertonic solutions are present in the intestinal lumen (28). It is speculated that lower albumin may be associated with disturbed absorption of nutrients or water loss due to diarrhea and with increased levels of various inflammatory markers, such as APPs. Given our results, diarrhea in calves was accompanied by a reduction in albumin, suggesting that low levels of albumin may indicate a higher risk of disease and poor health of animals.

Globulins comprise a much smaller protein component and are divided into 4 categories: α1-, α2-, β-, and γ-globulins. In this study, of the 4 globulin fractions, α2-globulins were present at significantly
higher levels in calves with diarrhea compared to normal calves. A previous study showed a significant association between diarrhea and α2-globulins in calves (21) and our findings are consistent with that result. The α2-globulins include some APPs, such as Hp, ceruloplasmin, and α2-macroglobulin, and are involved in inflammation (29). Diarrhea in calves usually causes inflammation of the intestines. As a result, the increase in α2-globulin supports the conclusion that inflammation had already developed in diarrheic calves.

The γ-globulin fraction is actively involved in the defense system against infectious agents (30,31). According to our results, overall γ-globulin concentrations were low in calves with diarrhea. The concentrations of γ-globulin also showed a decreasing trend with age in calves. The progressive decline of γ-globulin concentrations in normal calves may be attributed to the degradation of colostrum-derived immunoglobulins and a gradual initiation of production of immunoglobulins by their maturing immune system. Contrary to the explanation of this change in normal calves, the reason for γ-globulin reduction in calves with diarrhea may be the shift from γ-globulin to α2-globulins. As such, a decrease of γ-globulin may confer lower immune resistance when calves need protection from various infections, resulting in worsened clinical symptoms. Consequently, these results indicate that γ-globulin levels in serum may be influenced by the health status of calves.

In this study, the most marked difference between the 2 groups was in the concentrations of Hp. Haptoglobin concentrations in calves affected by diarrhea were more than 5-fold higher than in normal calves; in contrast, SAA levels did not exhibit changes with diarrhea. A previous study performed by our group showed that the concentrations of SAA increased in coronavirus-infected calves, but it was not to a statistically significant extent (10). The biggest difference between the current and previous experiments is the age of the calves used; consequently, with the results from the current study, SAA may be a less suitable diagnostic parameter for the assessment of calf health. Moreover, Hp has been used in detecting disease status and inflammation (32). In several studies, Hp concentrations in serum were found to be significantly increased in calves with respiratory diseases, suggesting that there were associations between serum Hp concentrations and respiratory tract disease (14,32–34). Angen et al (35) reported that although SAA concentrations increased in calves with respiratory disease, the values did not show a significant difference when compared to changes in Hp. This result was consistent with the present study. Haptoglobin is a good marker to identify calves with respiratory disease. The concentrations of Hp were significantly higher in calves with diarrhea, indicating that this phenomenon may be associated with increased α2-globulin concentrations. In addition, there was a positive correlation among Hp, α2-globulin, and diarrhea. Taken together, these results suggest that Hp may be important as a sensitive marker that is preferable in the field and can be useful for monitoring the disease status of animals because of its larger and more prolonged response.

The present study showed that changes in serum protein electrophoretic profile and Hp levels are markedly influenced by the health condition of calves. Significant differences were found for α2-globulin and γ-globulin in calves with diarrhea. Moreover, Hp concentrations in calves with diarrhea were significantly increased and revealed a positive correlation between α2-globulin and diarrhea. These results provide valuable information for clinicians who can use serum protein profiles and Hp to evaluate the diagnosis and prognosis of calves with diarrhea.

Figure 3. Correlation between haptoglobin (Hp) and α2-globulin. The concentrations of Hp were positively correlated with α2-globulin (P = 0.0004) in calves with diarrhea.

Acknowledgment

This work was supported by the Jeonbuk National University Promotion Development Project in 2019.

References


An initial genome-wide investigation of protein-losing enteropathy in Gordon setters: Exploratory observations

Elle K. Donnini, Muhammed Walugembe, Max F. Rothschild, Albert E. Jergens, Karin Allenspach

Abstract

The objective of this preliminary study was to identify genomic regions that may predispose Gordon setters from the United Kingdom to familial protein-losing enteropathy (PLE) at a young age. A total of 106 related Gordon setters was used, including 6 affected dogs from an affected litter, 6 case controls from the same litter, 10 related/affected dogs, and 84 related/unaffected dogs. Genomic DNA was collected from each Gordon setter and extracted from buccal mucosal swabs. Genotyping of affected and unaffected dogs was carried out using the Canine Illumina HD SNP array and data generated were analyzed with PLINK software, using fixation index (Fst) and runs of homozygosity (ROH) methods. Pairwise Fst analyses between the affected and unaffected Gordon setter dogs identified various regions of differentiation on chromosomes 10, 18, 21, and 23 that contained several important genes. These regions revealed 5 candidate genes, including RARB, TTC7A, SOCS5, PIGF, and RHOD, that are associated with human inflammatory bowel disease (IBD) and could potentially be associated with PLE in Gordon setters. Run of homozygosity (ROH) analyses revealed additional unique regions on chromosomes 15 and 17. These regions contained genes SYT1, UCN, and FNDC that could also be potential candidates for PLE in Gordon setters. The biological functions of the identified genes provided initial insights into the pathophysiology of PLE. Further large-scale studies are warranted to investigate the possible causality of these genomic regions and any possible genetic markers that could be used in predicting susceptibility to PLE syndrome.

Résumé

L’objectif de cette étude préliminaire était d’identifier les régions génomiques susceptibles de prédisposer les chiens Gordon setter du Royaume-Uni à l’entéropathie familiale de perte de protéines (PLE) à un jeune âge. Un total de 106 Gordon setter apparentés a été utilisé, dont six chiens affectés d’une portée affectée, six cas témoins de la même portée, 10 chiens apparentés/affectés et 84 chiens apparentés/non affectés. L’ADN génomique a été obtenu à partir de chaque Gordon setter et extrait des écouvillons de la muqueuse buccale. Le génotypage des chiens affectés et non affectés a été effectué à l’aide de la matrice SNP Canine Illumina HD et les données générées ont été analysées avec le logiciel PLINK, en utilisant des méthodes d’indice de fixation (Fst) et d’homozygotie (ROH). Des analyses Fst par paires entre les chiens Gordon setter affectés et non affectés ont identifié diverses régions de différenciation sur les chromosomes 10, 18, 21 et 23 qui contenaient plusieurs gènes importants. Ces régions ont révélé cinq gènes candidats, dont RARB, TTC7A, SOCS5, PIGF et RHOD, qui sont associés à la maladie inflammatoire de l’intestin (IBD) humaine et pourraient potentiellement être associés à la PLE chez les Gordon setter. Les analyses d’homozygotie (ROH) ont révélé des régions uniques supplémentaires sur les chromosomes 15 et 17. Ces régions contenaient les gènes SYT1, UCN et FNDC qui pourraient également être des candidats potentiels pour la PLE chez les Gordon setter. Les fonctions biologiques des gènes identifiés ont fourni un aperçu initial de la physiopathologie de la PLE. D’autres études à grande échelle sont nécessaires pour étudier la causalité possible de ces régions génomiques et tous les marqueurs génétiques possibles qui pourraient être utilisés pour prédire la sensibilité au syndrome PLE.

(Traduit par Docteur Serge Messier)
Introduction

Protein-losing enteropathy (PLE) in dogs is caused by disease of the small intestines that is severe enough to cause leakage of plasma proteins into the lumen of the gut. The major causes of PLE in dogs include mucosal infiltrative disease (inflammatory bowel disease, intestinal lymphoma, and gastrointestinal histoplasmosis) and intestinal lymphangiectasia (1). Systemic consequences of PLE are largely related to hypoproteinemia and include cavitary effusions, pitting edema, hyper-coagulability, and/or cachexia. Typically, dogs with PLE have a variable response to medical treatment and an uncertain prognosis depending on the underlying cause (2–5).

Although PLE can affect any dog, certain breeds have been described as predisposed, including the Basenji, Lundehund, Rottweiler, soft-coated wheaten terrier, Yorkshire terrier, and Chinese shar pei (3,6–9). A potential hereditary basis for PLE has been proposed in many of these breeds, although the underlying etiology remains unclear. In human medicine, hereditary factors mediating intestinal immunity, i.e., hypogammaglobulinemia and immunologic dysfunction of the intestinal mucosa, have been investigated and implicated in certain protein-losing diseases (10).

More recent literature has documented other immunologic deficits in humans with PLE, including impaired antibody function, B-cell depletion, T-cell depletion, and antibody depletion (IgG, IgA, IgM), although a hereditary basis for these defects in intestinal immunity has not been recognized (11). While similar studies are lacking in veterinary medicine, depletion in local gut immunologic factors and a hereditary basis for loss of intestinal protein in certain breeds are strongly suspected.

Genome-wide association studies (GWAS) are an emerging diagnostic tool in veterinary medicine and offer a unique opportunity to screen for genomic regions and candidate loci that contribute to susceptibility for heritable diseases. Such an approach differs from candidate gene analysis in that it evaluates the entire genome for common genetic variation, while the candidate gene approach focuses on associations between genetic variants within pre-specified genes of interest. Genome-wide association studies (GWAS) have been used to investigate a hereditary basis for several diseases in dogs, including inflammatory bowel disease (IBD) in German shepherd dogs, ectopic ureters in Entlebucher mountain dogs, mitral valve disease in Maltese dogs, and syringomyelia in Cavalier King Charles spaniels (12–14).

Focusing specifically on a hereditary basis for chronic enteropathies in German shepherds, 16 candidate genes that contribute to phenotypic expression of inflammatory bowel disease have been identified using a GWAS (12). Other veterinary literature suggests a hereditary basis for chronic enteropathies in other breeds and supports the diagnostic usefulness of GWAS in identifying an underlying etiology for PLE (8,9,15). Furthermore, recent GWAS in humans have identified over 200 risk loci that confer increased susceptibility on individuals to develop IBD (16–18). Collectively, these studies further illustrate the potential for GWAS to aid in identifying a heritable basis for complex genetic diseases.

This preliminary study involved a family of 16 related Gordon setters diagnosed with PLE based on compatible clinical signs and variable diagnostic investigations. The control group consisted of 90 related Gordon setters with no evidence of clinical signs. The aim of the current exploratory study was to detect possible genomic regions and single nucleotide polymorphisms (SNPs) that might be associated with PLE in a limited but defined Gordon setter population using a genome-wide association (GWA) approach. To the authors' knowledge, this is the first genome-wide association study (GWAS) of both healthy and affected Gordon setters with the aim of investigating SNPs that may be significantly associated with the phenotype of PLE.

Materials and methods

Study population

A total of 106 related Gordon setters was included retrospectively in this study; those included were categorized as either “affected” or “unaffected.” “Affected” dogs displayed a wide range of clinical signs, including vomiting, diarrhea, inappetence, and inability to gain/maintain weight. All owners of participating dogs signed a consent sheet for obtaining buccal mucosal swabs for genotyping and filled in a questionnaire related to their dogs’ health.

The study population originated from a litter of Gordon setter puppies (n = 12) in the United Kingdom in which a disproportionate number of puppies (n = 6) was affected by the previously mentioned clinical signs. The other half of the identified litter (n = 6) displayed no clinical signs consistent with PLE. A total of 94 related Gordon setters was also identified from around the world. Of these 94 dogs, 10 were identified as having clinical signs consistent with PLE and...
Table I. Genes in regions of possible genomic differentiation from fixation index (Fst) analysis for comparison of affected versus unaffected Gordon setter dogs.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position window (Mb)</th>
<th>Gene</th>
<th>Gene name</th>
<th>Chromosome</th>
<th>Position window (Mb)</th>
<th>Gene</th>
<th>Gene name</th>
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<td>48840243-48851461</td>
<td>CRIPT</td>
<td>CXCR repeat containing interactor of PDZ3 domain</td>
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<td>50676138-50686061</td>
<td>RBM4B</td>
<td>RNA-binding motif protein 4B</td>
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<td>10</td>
<td>48746331-48754047</td>
<td>ATP6V1E2</td>
<td>ATPase H+ transporting V1 subunit E2</td>
<td>18</td>
<td>50748039-50759270</td>
<td>CCS</td>
<td>Copper chaperone for superoxide dismutase</td>
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<tr>
<td>10</td>
<td>49102256-49246663</td>
<td>TTC7A</td>
<td>Tetrameric repeat domain 7A</td>
<td>18</td>
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<td>LOC610648</td>
<td>RNA-binding protein 14</td>
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<td>10</td>
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<td>MCFD2</td>
<td>Multiple coagulation factor deficiency 2</td>
<td>18</td>
<td>50621518-50632044</td>
<td>LOC102157139</td>
<td>pH-response regulator protein pall/pr-5-like</td>
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<td>10</td>
<td>48916446-48977183</td>
<td>SOCS5</td>
<td>Suppressor of cytokine signaling 5</td>
<td>21</td>
<td>47349412-47362174</td>
<td>MUC15</td>
<td>Mucin 15, cell surface-associated</td>
</tr>
<tr>
<td>10</td>
<td>48804226-48840188</td>
<td>PIGF</td>
<td>Phosphatidylinositol glycan anchor biosynthesis class F</td>
<td>21</td>
<td>47671719-47674127</td>
<td>FIBIN</td>
<td>Fin bud initiation factor homolog (zebrafish)</td>
</tr>
<tr>
<td>10</td>
<td>48766623-48807198</td>
<td>RHOQ</td>
<td>Ras homolog family member Q</td>
<td>21</td>
<td>47707648-47766732</td>
<td>BBOX1</td>
<td>Gamma-butyrobetaine hydroxylase 1</td>
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<td>18</td>
<td>50394024-50395968</td>
<td>C18H11orf86</td>
<td>Chromosome 18 C11orf86 homolog</td>
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<td>47421535-47466664</td>
<td>SLC5A12</td>
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<td>18</td>
<td>50512681-50617666</td>
<td>C18H11orf80</td>
<td>Chromosome 18 C11orf80 homolog</td>
<td>21</td>
<td>47046020-47416360</td>
<td>ANO3</td>
<td>Anoctamin 3 (Canis lupus familiaris)</td>
</tr>
<tr>
<td>18</td>
<td>50320428-50330108</td>
<td>RHOD</td>
<td>Ras homolog family member D</td>
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<td>18207253-18931128</td>
<td>RARB</td>
<td>Retinoic acid receptor beta</td>
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<tr>
<td>18</td>
<td>50495971-50499062</td>
<td>LRFN4</td>
<td>Leucine-rich repeat and fibronectin type-III domain-containing 4</td>
<td>23</td>
<td>18044479-18101937</td>
<td>NGLY1</td>
<td>N-glycanase 1</td>
</tr>
<tr>
<td>18</td>
<td>50631053-50670428</td>
<td>SPTBN2</td>
<td>Spectrin beta, non-erythrocytic 2</td>
<td>23</td>
<td>18159521-18206413</td>
<td>TOP2B</td>
<td>DNA topoisomerase II beta</td>
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<td>RCE1</td>
<td>Ras-converting CAAX endopeptidase 1</td>
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<td>18034532-18045630</td>
<td>OXSM</td>
<td>3-oxoacyl-ACP synthase, mitochondrial</td>
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<td>18</td>
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<td>PC</td>
<td>Pyruvate carboxylase</td>
<td>23</td>
<td>17873707-17874136</td>
<td>LOC610034</td>
<td>Ribosomal protein L31 pseudogene</td>
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<td>18</td>
<td>50334779-50358113</td>
<td>SYT12</td>
<td>Synaptotagmin 12</td>
<td>23</td>
<td>19278650-19648547</td>
<td>THRBJ</td>
<td>Thyroid hormone receptor beta</td>
</tr>
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<td>18</td>
<td>50178511-50294097</td>
<td>KDM2A</td>
<td>Lysine demethylase 2A</td>
<td>23</td>
<td>19729208-19729660</td>
<td>LOC102153140</td>
<td>60S ribosomal protein L26-like</td>
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</table>
84 dogs were apparently healthy based on an owner questionnaire. Buccal mucosal swabs were obtained for all 106 Gordon setters included in the study and submitted for DNA extraction. Iowa State University’s Institutional Animal Care and Use Committee (IACUC) determined that official ethics approval was not needed as the samples were buccal mucosal swabs and all owners had given informed consent.

**Genotyping and quality control**

Genomic DNA were extracted from buccal swabs using standard approaches. All controls and cases were genotyped using high density CanFam2.0 BeadChip (Illumina, San Diego, California, USA). Genotype data was filtered for quality control with PLINK, version 1.9 software (19) and included a single nucleotide polymorphism (SNP) call rate of $\geq 90\%$ and a minor allele frequency of $\geq 0.02$. A total of 119 870 SNPs remained after genotyping and filtering and these were used for the downstream analyses.

**Fixation index (Fst) analyses**

Two pairwise comparisons were conducted for all case versus control dogs and case versus control siblings using a sliding genomic window approach in PLINK v1.9 (19) to identify any genomic regions under increasing differentiation. For the comparison, the mean fixation index (mFst) value was calculated in 500-kb sliding windows with a step size of 250 kb to examine a 50% overlap using an in-house script (20). Suggestive windows with highest peaks of $> 0.2\%$ of the empirical distribution of mFst values were used for downstream analyses.

**Runs of homozygosity (ROH)**

Genomic regions of high homozygosity were analyzed separately in the controls and cases and conducted with the --homozyg command in PLINK v1.9 (19). For each window, 50 SNPs were allowed (homozyg-window-snp 50), 1 missing call (homozyg-window-missing 1), and 3 heterozygotes (homozyg-window-het 3). To normalize, a raw value for each SNP in the summary output file (plink.hom.summary) indicating the number of animals, was divided by the number of animals that were included in the analyses, which allowed us to compute locus homozygosity (21). Locus homozygosity ($H$) ranged from 0 (0%) to 1 (100%) and regions with $H \geq 0.7$ at each SNP location were considered as the regions of high homozygosity.

**Gene annotation**

Genomic regions with the highest mFst and homozygosity were annotated using the National Center for Biotechnology Information’s (NCBI) Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) for Canis lupus familiaris (dog).

**Results**

**Demographics of study population**

Affected dogs were identified, including 6 affected littermates and 10 additional, related affected dogs, based on clinical signs and variable diagnostic investigations. The most commonly reported clinical signs included chronic diarrhea (13/16, 81.25%), weight loss (12/16, 75%), vomiting (1/16, 6.25%), and panhypoproteinemia (16/16, 100%). Other reported biochemical abnormalities included hypocobalaminemia (13/15, 95%), anemia (1/4, 25%), low folate (1/4, 25%), hypocholesterolemia (12/13, 75%), and elevated partial thromboplastin time (PTT) (1/4, 25%). Abdominal ultrasounds were carried out on 12 dogs and revealed moderate amounts of peritoneal effusion (12/12), intestinal wall thickening (12/12), and slightly increased size of mesenteric lymph nodes (3/12). Intestinal biopsies and a histopathologic diagnosis of PLE were available for 13/16 dogs (i.e., all 6 of the affected puppies and 7/10 of the related adult affected dogs).

Histopathology of small intestinal biopsies or necropsy revealed lymphoplasmacytic plus eosinophilic inflammation ($n = 10$), followed by lymphoplasmacytic plus neutrophilic inflammation ($n = 9$ duodenum, $n = 6$ ileum). Crypt abscessation or dilation was found in 12/13 dogs in the duodenum and lacteal dilation in 13/13 cases.

Dogs were treated using various different therapies, including oral steroids (6/13, 46%), a hydrolyzed protein diet (13/13, 100%), metronidazole (1/13, 8%), cyclosporine (1/13, 8%), and cobalamin injections (15/16, 94%). All puppies were euthanized within 6 wk after diagnosis of intractable diarrhea. The adult affected dogs were euthanized 6 to 12 wk after diagnosis due to intractable disease.

**Fixation index (Fst) analyses**

Fixation index (Fst) analyses for the comparison of all affected ($n = 16$) versus unaffected dogs (all healthy, $n = 90$) indicated a strong
### Table II. Runs of homozygosity significant in affected Gordon setter dogs.

<table>
<thead>
<tr>
<th>Chromosome: Window size</th>
<th>Number of SNPs</th>
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<td>10: 5097479-5977990</td>
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<td>TAPA (TAPA chemokine-like family member 2)</td>
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<td></td>
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<td>MON2 (MON2 homolog, regulator of endosome-to-Golgi trafficking)</td>
<td>5670388-5776533</td>
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<td></td>
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<td>PPM1H (protein phosphatase, Mg2+/Mn2+ dependent 1H)</td>
<td>5818660-6072182</td>
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<tr>
<td></td>
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<td>USP15 (ubiquitin-specific peptidase 15)</td>
<td>5510759-5634954</td>
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<tr>
<td>15: 20000745-20999682</td>
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<td>NAV3 (neuron navigator 3)</td>
<td>19931037-20937244</td>
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<td>15: 21009206-21994974</td>
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<td>SYT1 (synaptotagmin 1)</td>
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<td>TRNAR-CCU [transfer RNA arginine (anticodon CCU)]</td>
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<td>PAWR (pro-apoptotic WT1 regulator)</td>
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<td>LOC1010684031 (peptidyl-prolyl cis-trans isomerase FKBP3 pseudogene)</td>
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<td>MPV17 (mitochondrial inner membrane protein MPV17)</td>
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<td>SUPT7L (SPT7-like, STAGA complex gamma subunit)</td>
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<td>ZNF512 (zinc finger protein 512)</td>
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<td>CFAP100 (cilium- and flagella-associated protein 100)</td>
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<td>TNXRD3 (thioredoxin reductase 3)</td>
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<td>CHST13 (carbohydrate sulfotransferase 13)</td>
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<td>PLXNA1 (plexin A1)</td>
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</tbody>
</table>
peak on chromosome 10 (mFst > 0.2) (Figure 1) that contained 3 windows (4.8 to 5.3 Mb; 5.3 to 5.8 Mb; and 5.8 to 6.3 Mb) and many genes underlying these regions (Table I). The comparison between case (affected) and control (unaffected) siblings further revealed high peaks on chromosomes 10, 18, 21, and 23 (Figure 2). A total of 7 regions was detected on chromosomes 10 (48.8 to 49.3 Mb), 18 (50.3 to 50.8 Mb), 21 (47.3 to 47.8 Mb), and 23 (17.8 to 18.3 Mb, 18.3 to 18.8 Mb, 18.8 to 19.3 Mb, and 19.3 to 19.8 Mb). The regions on chromosomes 10 and 23 contained several possibly important genes, including RARB on chromosome 10 and TTC7A, SOCS3, PIGF, and RHOD on chromosome 23.

Runs of homozygosity

Runs of homozygosity (ROH) were separately analyzed for affected and unaffected groups of Gordon setter dogs. Regions of homozygosity with $H > 0.7$ that were present in the case (affected) group but absent in the control (unaffected) group, and vice versa, were considered for gene ontology analyses. The average length of the identified regions using the selected option “homozyg-window-snp” of 50 SNPs was $\leq 1$ Mb. Fifteen regions were identified on chromosomes 10, 15, 17, 18, 19, 20, and 23 in the affected Gordon setter dogs (Table II; Figure 3). One region was detected (from 14171011 to 15853047 bp) with the highest $H$ value of 0.89 located on chromosome 23. For the unaffected Gordon setter sibling group, 3 regions of homozygosity were detected on chromosomes 12 and 18 (Figure 3), with the highest $H$ value (from 47861674 to 48996270) of 0.8 detected on chromosome 18. The annotated genes in the high homozygosity regions for the affected and unaffected Gordon setter dogs are listed in Tables II and III, respectively. For each of the annotated genes, the $H$ value was the same from the start to the end SNPs of the gene.

Table II. Runs of homozygosity significant in affected Gordon setter dogs (continued).

<table>
<thead>
<tr>
<th>Chromosome: Window size</th>
<th>Number of SNPs</th>
<th>Gene name</th>
<th>Gene location</th>
</tr>
</thead>
<tbody>
<tr>
<td>20: 2003229-2994432</td>
<td>40</td>
<td>RAB7A (RAB7A, member RAS oncogene family)</td>
<td>2704716-2776518</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SEC61A1 (Sec61 translocon alpha 1 subunit)</td>
<td>2070739-2087790</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATA2 (GATA-binding protein 2)</td>
<td>2472251-248835</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAB43 (RAB43, member RAS oncogene family)</td>
<td>2979094-3020496</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNAJB8 [DnaJ heat shock protein family (Hsp40) member B8]</td>
<td>2453100-2454152</td>
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<tr>
<td></td>
<td></td>
<td>ISY1 (ISY1 splicing factor homolog)</td>
<td>2945636-2972245</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMCMES (5-hydroxymethylcytosine-binding, ES cell-specific)</td>
<td>2808867-2831123</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H1FX (H1 histone family, member X)</td>
<td>2799360-2800524</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KBTBD12 (kelch repeat and BTB domain-containing 12)</td>
<td>1955638-203435</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CNBP (CCCH-type zinc finger nucleic acid-binding protein)</td>
<td>2860915-2929307</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COPG1 (coatomer protein complex subunit gamma 1)</td>
<td>2832150-2860356</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPN1 (ribophorin I)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>EEFSEC (eukaryotic elongation factor, selenocysteine-tRNA-specific)</td>
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<td></td>
<td></td>
<td>RUVBL1 (RuB-like AAA ATPase 1)</td>
<td>2095807-2137528</td>
</tr>
<tr>
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<td></td>
<td>LOC111091356 (heat (basic salivary proline-rich protein 2-like)</td>
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<tr>
<td>23: 15015702-15853047</td>
<td>41</td>
<td>ZCWPW2 (zinc finger CW-type and PWWP domain-containing 2)</td>
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<td>1982562-19829807</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR1D2 — nuclear receptor subfamily 1 group D member 2</td>
<td>19758907-19787725</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UBE2E1 — ubiquitin-conjugating enzyme E2 E1</td>
<td>19863136-19937272</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NKIRAS1 — NFkB inhibitor interacting Ras-like 1</td>
<td>19829764-19861462</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LOC106557641 — cell division cycle-associated protein 3 pseudogene</td>
<td>19921842-19926274</td>
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<td>UBE2E2 — ubiquitin-conjugating enzyme E2 E2</td>
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<td></td>
<td></td>
<td>LOC111091908 — proline-rich protein 18-like</td>
<td>20677483-20718136</td>
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<tr>
<td>23: 21013425-21208233</td>
<td>16</td>
<td>ZNF385D — zinc finger protein 385D</td>
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</table>

SNPs — single nucleotide polymorphisms.
Regions with low heterozygosity.

A threshold of 0.7, indicated by a red line, was chosen to detect

Figure 3. Regions of homozygosity in affected (A) and unaffected (B) dogs. A threshold of 0.7, indicated by a red line, was chosen to detect regions with low heterozygosity.

Discussion

In this study, a litter of Gordon setters from the United Kingdom affected with a possibly inheritable form of PLE is described. The genome-wide association (GWA) approach was applied to 106 related Gordon setters, including 6 affected dogs from an affected litter, 6 case controls from the same litter, 10 related/affected dogs, and 84 related/unaffected dogs, identifying 8 possible genes that could be implicated in the disease. This is a preliminary study limited in part by the collection of both affected dogs and their relatives. The initial Fst results indicated regions on chromosomes 10, 18, 21, and 23 under possible differentiation between affected and unaffected Gordon setter dogs (Table I).

Five genes in the regions of differentiation have previously been implicated in phenotypic expression of different gastrointestinal diseases in humans. These 5 genes are: TTC7A, SOCS5, and PIGF on chromosome 10; RHOD on chromosome 18; and RARB on chromosome 23. As a group, these genes encompass a wide variety of functions, including gastrointestinal immune function, cancer suppression, intestinal development, protein synthesis and packaging, inflammatory mediators, and regulating angiogenesis.

A region of differentiation on chromosome 23 contained a gene, RARB, that is important in cell growth and development. RARB encodes for the protein retinoic acid receptor-beta, which is a member of the thyroid-steroid hormone receptor family of nuclear transcription regulators. This receptor binds retinoic acid, an important molecule in mediating embryologic cell morphology, growth, and differentiation (22). In human medicine, RARB was first identified as a tumor-suppressor gene in colonic wall neoplasms (22). Mutations (hypermethylation) in the RARB promoter region have since been linked to the development of mammary cancer, lung cancer, and prostatic cancer (23–26). The mechanisms underlying hypermethylation of tumor-suppressor, gene-promoter regions are poorly understood, but ultimately this hypermethylation is thought to alter chromatin structure, which leads to silencing of the tumor-suppressor gene.

Interestingly, a more recent human study found hypermethylation of the promoter region of the RARB gene and subsequent downregulation of messenger RNA (mRNA) production from epithelial cells from inflamed mucosa in patients with ulcerative colitis (27). Although RARB has historically been identified as a tumor-suppressor gene, this was the first study suggesting hypermethylation and downregulation of the RARB gene with non-neoplastic intestinal inflammation. Future studies involving a larger number of dogs are warranted in order to look for similar mutations in the RARB gene, particularly in cases of colonic neoplasia and idiopathic inflammatory bowel disease (IBD). It is unclear whether RARB SNP mutations play a role in the development of PLE in this population of affected dogs.

It has been reported that the genes TTC7A and SOCS5 play a role in regulating the immune system in cases of chronic intestinal inflammation in humans. An increasing incidence of early onset IBD in humans, in combination with increased efficiency of whole-genome sequencing, has led to the identification of an autosomal recessive combined syndrome of immunodeficiency (T- and B-cell) secondary to TTC7A deficiency. TTC7A acts as a scaffolding protein that binds and recruits phosphatidylinositol 4-kinase III alpha to the plasma membrane, facilitating production of PI4-phosphate (PI4P) (28). Humans with TTC7A mutations manifest a combination of intestinal and immunologic diseases, including multiple intestinal atresias, early onset IBD, loss of intestinal architectural, apoptotic enterocolitis, combined immunodeficiency syndrome, and various other diseases of the hair and skin (29–32). The gene SOCS5 is a member of the cytokine-suppressor gene family, with important immune regulatory activities. Deficiency of cytokine-suppressor genes has been documented in both human and murine models (33,34). Similar immune dysfunction and gastrointestinal disease may exist in Gordon setters and other canine breeds with TCC7A and SOCS5 mutations. Importantly, this is the first study to suggest differentiation of affected versus unaffected dogs in the genomic regions containing the TCC7A and SOCS5 genes.

The gene, RHOD (ras homolog family member D), belongs to a family of structural signaling proteins (superfamily ras). Accumulating evidence implicates small G proteins of the Ras superfamily as important signaling molecules for regulating epithelial junctions (35,36) that may lead to altered gastrointestinal structure, functional abnormalities, and expression of an IBD/PLE phenotype in affected dogs. Angiogenesis also plays an important role in the development of inflammatory bowel disease and placental growth factor (PIGF) is an important regulator of pathologic angiogenesis (37,38). Up-regulation of PIGF and subsequent pro-inflammatory effects have been documented in human cases of inflammatory bowel disease (38). While up-regulation of PIGF has
not been documented in cases of IBD/PLE in veterinary medicine, PIGF polymorphisms in this study were associated with the PLE phenotype in Gordon setters. Regions of high homozygosity were explored in both affected and unaffected Gordon setter dogs across the dog genome looking for possible deleterious mutations. We considered the $H$ value threshold of $H > 0.7$ to account for the presence of any mutations, possible genotyping errors, and assembly errors that could locate an SNP in a wrong position of the genome. High ROH regions were identified on chromosomes 10, 15, 17, 18, 19, 20, and 23. Several genes were annotated in these regions that have been implicated in phenotypic expression of different gastrointestinal diseases in humans, including SYT1, UCN, and FNDC4 (Table II). The gene, SYT1 (syntotagmin 1), encodes for a transmembrane vesicular transport protein and belongs to a family of calcium sensors responsible for releasing certain neurotransmitters at the synapse (34).

### Table III. Runs of homozygosity significant in unaffected Gordon setter dogs.

<table>
<thead>
<tr>
<th>Chromosome: Window size of SNPs</th>
<th>Gene name</th>
<th>Gene location</th>
</tr>
</thead>
<tbody>
<tr>
<td>18: 47861674-47991229 14</td>
<td>CTTN (cortactin)</td>
<td>47932367-47960732</td>
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<tr>
<td></td>
<td>SHANK2 (SH3 and multiple ankyrin repeat domains 2)</td>
<td>47477625-47915557</td>
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<tr>
<td></td>
<td>PPIA1 (PTPRF interacting protein alpha 1)</td>
<td>47974109-48060331</td>
</tr>
<tr>
<td>18: 48008717-48996270 72</td>
<td>CCND1 (cyclin D1)</td>
<td>48501905-48509684</td>
</tr>
<tr>
<td></td>
<td>FGF4 (fibroblast growth factor 4)</td>
<td>48413480-48417494</td>
</tr>
<tr>
<td></td>
<td>ANO1 (anocamin 1)</td>
<td>48108310-48249021</td>
</tr>
<tr>
<td></td>
<td>LTO1 (LTO1, ABCE1 maturation factor)</td>
<td>48428212-48490678</td>
</tr>
<tr>
<td></td>
<td>TPCN2 (two-pore segment channel 2)</td>
<td>48956833-48984499</td>
</tr>
<tr>
<td></td>
<td>FG19 (fibroblast growth factor 19)</td>
<td>48464931-48469967</td>
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<tr>
<td></td>
<td>PPIA1 (PTPRF interacting protein alpha 1)</td>
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<tr>
<td></td>
<td>FADD (Fas associated via death domain)</td>
<td>48087447-48094497</td>
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<tr>
<td></td>
<td>FGF3 (fibroblast growth factor 3)</td>
<td>48382819-48388453</td>
</tr>
<tr>
<td>18: 49014221-49992069 84</td>
<td>GSTP1 (glutathione S-transferase pi 1)</td>
<td>49905161-49908182</td>
</tr>
<tr>
<td></td>
<td>GALT (galanin and GMAP prepropeptide)</td>
<td>49240437-49246543</td>
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<tr>
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<td>CPT1A (carnitine palmitoyltransferase 1A)</td>
<td>49136691-49190117</td>
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<td>MRGPRD (MAS-related GPR family member D)</td>
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<td>MRGPRF (MAS-related GPR family member F)</td>
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<td>C18H11orf24 (chromosome 18 C11orf24 homolog)</td>
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<td>LRP5 (LDL receptor-related protein 5)</td>
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<td>TESMIN (testis-expressed metallothionein-like protein)</td>
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<td>AIP (aryl hydrocarbon receptor interacting protein)</td>
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<td>PTPN1M1 (phosphatidylinositol transfer protein membrane-associated 1)</td>
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<tr>
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<td>CDK2AP2 (cyclin-dependent kinase 2-associated protein 2)</td>
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<tr>
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<td>NUDT8 (nudix hydrolase 8)</td>
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<td>NDUF58 (NADH:ubiquinone oxidoreductase core subunit S8)</td>
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<tr>
<td></td>
<td>UNC93B1 (unc-93 homolog B1, TLR signaling regulator)</td>
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<td>TLRG1 (T cell immune regulator 1, ATPase H+ transporting V0 subunit a3)</td>
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<tr>
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<td>KMT5B (lysine methyltransferase 5B)</td>
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<td>PPP6R3 (protein phosphatase 6 regulatory subunit 3)</td>
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<td>IGHMBP2 (immunoglobulin mu DNA-binding protein 2)</td>
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<td>MRPL21 (mitochondrial ribosomal protein L21)</td>
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<td>ACY3 (aminoacylase 3)</td>
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</tr>
<tr>
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<tr>
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<td>LOC476006 (glutathione S-transferase P-like)</td>
<td>49950873-49953856</td>
</tr>
<tr>
<td></td>
<td>TRNA-AGC [transfer RNA alanine (anticodon AGC)]</td>
<td>49432968-49433040</td>
</tr>
</tbody>
</table>

SNPs — single nucleotide polymorphisms.
secondary to inflammation induced by lipopolysaccharide (LPS) (39). Induction of this “reactive human enteric glial cell” phenotype with LPS disrupted calcium signaling and led to alterations in other important molecular and functional signaling pathways, such as mechanosensory and purigenic. Although mutations in STY1 and other members of this vesicular transport protein family were not found in this study, alterations in the expression of vesicular transport proteins could result in altered gastrointestinal motility and clinical signs consistent with protein-losing enteropathy. This study in human medicine, however, implied a change in expression of vesicular membrane protein secondary to inflammation, not inflammation caused by altered expression of STY1 (39).

Urocortin, a protein that is encoded for by the UCN gene, belongs to the corticotropin-releasing factor family of proteins and is important in regulating the mammalian stress response and appetite. Human data have documented that disrupted signaling of urocortin-dependent corticotropin-releasing hormone 2 can alter gastrointestinal epithelial cell differentiation (40). These changes could be relevant to the intestinal epithelial alterations found in IBD in both humans and dogs (40). Additionally, a murine study found that treatment with urocortin ameliorated clinical and histopathologic severity of inflammatory colitis by downregulating inflammation driven by T-helper 1 cells (41). FNDC4 (fibronectin type-III domain-containing 4) encodes for another protein with anti-inflammatory properties and is significantly elevated in cases of human IBD (42). Findings were similar to those for the UCN gene for up-regulation of FNDC4 in reducing severity of clinical and histopathologic severity of colitis in mice (43). These studies illustrate how UCN and FNDC4 mutations may contribute to the development of a PLE/IBD phenotype in dogs and also highlight an unexplored therapeutic strategy in canine patients with PLE.

Collectively, the 8 candidate genes preliminarily identified in this initial study illustrate the multi-factorial and complex pathogenesis of IBD/PLE in dogs and humans. Our study also helps to point out the possible usefulness of fixation index (Fst) and ROH analysis methodologies in identifying desired genetic variation in research colonies used as models for human drug testing.

This study had some limitations. Although a total of 106 dogs was included in this study, our analyses focused on the affected and unaffected siblings in order to examine genome-wide differentiations between affected and unaffected Gordon setter dogs and explore ROH regions within each group. Additional groups of full siblings that included both affected and unaffected dogs would have been quite useful. Furthermore, histopathological diagnosis of PLE secondary to IBD only occurred in 13/16 cases, although the remaining cases displayed chronic diarrhea, panhypoproteinemia, peripheral edema, and ascites, all of which are hallmarks of PLE in dogs. Finally, our study evaluated animals using an SNP chip across the genome. This approach may have missed rare alleles that may have contributed to the phenotypic expression of PLE in this population of related dogs, but were not on the chip.

In conclusion, this study identified 8 possible candidate genes that may contribute to the phenotypic expression of familial PLE in Gordon setters. Some of these genes may also provide information for future therapeutic options in canine patients with PLE via anti-inflammatory targets on a cellular level.

References


Treatment of naturally occurring asthma with inhaled fluticasone or oral prednisolone: A randomized pilot trial

Michael Verschoor-Kirss, Elizabeth A. Rozanski, Claire R. Sharp, Trisha J. Oura, Ashley Egan, Perry Bain, Joyce Knoll

Abstract

The objective of this study was to compare inhaled glucocorticoids with oral glucocorticoids for treatment of naturally occurring feline asthma. Secondary goals were to evaluate serum allergy testing results in cats and to quantify the effect of an inhaled glucocorticoid (fluticasone) on glucose homeostasis. Nine cats with asthma were enrolled on the basis of clinical signs, thoracic radiographic findings, and airway eosinophilia. Cats were randomized and 4 cats were treated with oral glucocorticoids and 5 cats with inhaled glucocorticoids, with a 7-day course of oral glucocorticoids overlapping at the start of therapy. Cats were evaluated at baseline and at 8 wk with thoracic radiographs, bronchoalveolar lavage, lung function testing, and fructosamine levels. Serum allergen panels were evaluated. All cats were clinically normal after treatment and had significantly improved airway eosinophilia and decreased nucleated cell count. No improvement was seen in radiographic changes after treatment with either therapy. Oral, but not inhaled glucocorticoids, caused a decrease in airway resistance, although cats in the inhaled group had a higher baseline resistance than those in the oral group. Fructosamine levels did not change with treatment. Fifty percent of cats tested positive for immunoglobulin E (IgE) antibodies. Asthma is a heterogeneous condition; individual cats responded well to both oral and inhaled glucocorticoids. Ongoing evaluation of the potential underlying causes and therapeutic options is warranted with a larger group of cats.

Résumé

L’objectif de l’étude était de comparer le traitement de l’asthme félin avec des glucocorticoides inhalés et administrés par voie entérale. Les objectifs secondaires étaient d’évaluer les résultats de tests d’allergies de chats atteints d’asthme félin et de quantifier l’effet d’un glucocorticoidé inhalé (fluticasone) sur l’homéostasie du glucose. Neuf chats atteints d’asthme félin ont été recrutés selon les signes cliniques, les trouvailles radiographiques et les évaluations cytologiques des voies aériennes (éosinophilie). Les chats ont été randomisés. Quatre chats ont été traités avec des glucocorticoides par voie entérale et cinq chats avec des glucocorticoides inhalés dont les 7 premiers jours ont été associés à l’administration de glucocorticoides par voie orale. Les chats ont initialement été évalués au moment du recrutement et puis à huit semaines avec des radiographies thoraciques, lavage bronchoalvéolaire, tests de fonction pulmonaire et dosage de la fructosamine. Des tests sériques d’allergènes ont également été évalués. Tous les chats ont eu une résolution des signes cliniques après le traitement et avaient une amélioration significative du compte éosinophilique du LBA. Aucune amélioration des lésions radiographiques suivant le traitement soit inhalé ou entéral n’a été observée. Seuls les glucocorticoides entéraux ont causé une diminution de la résistance des voies respiratoires. Toutefois les chats du groupe de traitement de glucocorticoides inhalés avaient, avant l’initiation du traitement, une résistance pulmonaire plus importante. Les niveaux de fructosamine n’ont pas changé significativement, et ce dans les deux groupes de traitement. 50 % des chats ont testé positif pour des anticorps IgE contre des allergènes inhalés communs. L’asthme est une entité clinique hétérogène; les chats ont individuellement bien répondu au traitement inhalé qu’au traitement entéral. L’étude des potentielles causes sous-jacente et des différentes options thérapeutiques sont recommandées dans une population plus grande de chats.

Introduction

Feline asthma is estimated to affect up to 5% of cats (1). Feline asthma is similar to human asthma in many ways, including bronchial thickening, airway hyperreactivity and remodeling, excess mucus production, cough, and occasional respiratory distress (1–2). While the underlying cause of feline asthma is unknown, the disease may be mimicked by sensitization to Bermuda grass allergen (BGA) or Ascaris suum, which supports an allergic etiology (3). Asthma is diagnosed in a cat with a cough, wheeze, and/or respiratory distress by excluding other potential causes of these clinical signs, such as heartworm or lung worm infestation, heart failure, infection, or neoplasia (4). Diagnostic evaluation for affected cats includes thoracic radiographs, which typically document a bronchial pattern,
and bronchoalveolar lavage (BAL) cytology, which demonstrates airway inflammation and eosinophilia (4). Treatment is directed at ameliorating airway inflammation through glucocorticoids (GCs) and rescue bronchodilators, as well as avoidance of potential triggers (5–7).

Glucocorticoids (GCs) are often administered orally in the form of prednisolone, although they can also be administered by inhalation or as a repository, e.g., methylprednisolone acetate, or transdermal preparations. Repository and transdermal medications are more commonly reserved for cats that may be harder to medicate. The efficacy of transdermal glucocorticoids has not been established.

Potential side effects of systemic oral glucocorticoids (O-GCs) include alterations in the hypothalamic-pituitary-adrenal (HPA) axis and derangements in glucose homeostasis, resulting in diabetes mellitus (DM), weight gain, and heart failure. It is thought that these adverse effects are minimized with the use of inhaled glucocorticoids (I-GCs) rather than O-GCs. Inhaled-GCs are most commonly used in humans as it has been demonstrated that delivering the therapeutic agent directly to the lungs is superior for long-term control of airway inflammation in humans with asthma (8).

Administering I-GCs to small children and domestic cats, however, requires the use of a spacer, as they are unable to follow instructions to use an inhaler directly. Due to the intrinsic differences between the nasal anatomy of a cat and a human, there are some disadvantages to the use of spacers with cats. Spacers may limit the dose reaching the lungs, as a certain percentage is swallowed rather than inhaled and some is also lost to the environment (9). This potentially limits the direct benefit of I-GCs and may negate any perceived GC-sparing benefit. Getting a cat to cooperate for aerosol administration and the prohibitive cost of inhaled GCs in the United States are other limitations to their use.

The best approach to therapy with glucocorticoids (GCs) for feline asthma is not known. The 2 common treatment approaches are oral prednisolone tapered to a dose low enough to control apparent clinical signs of cough, while minimizing side effects, or daily administration of inhaled GCs. It has been shown that resolution of clinical signs in humans is not universally associated with resolving airway inflammation and airway hyperreactivity (10). Similarly, evaluating the response to therapy for asthma in cats is challenging as the apparent resolution of clinical signs is not always associated with resolution of airway inflammation (11).

Unchecked airway inflammation may lead to progressive airway remodeling, excessive mucus production, and airway smooth muscle hypertrophy, with subsequent air-trapping and expiratory flow limitation. In humans, evaluation of lung function, specifically the forced expiratory volume in 1 s (FEV1), is widely used to better characterize expiratory flow limitation and response to therapy. While voluntary maximal expiratory effort is not possible in cats, similar information about flow limitation may be evaluated with flow-volume loops (12,13) and measurement of lung resistance (2,14). Radiographic improvement in response to treatment has been documented in cats with experimental asthma, but has not yet been evaluated in naturally occurring asthma (6).

Experimentally, Reinero et al (3) have recreated an asthma phenotype by sensitizing cats to Bermuda grass, while Kirshvink’s group created similar airway changes following exposure to A. suum (6). Inhaled glucocorticoids (I-GCs) have a beneficial effect on decreasing airway eosinophilia mediated by exposure to Bermuda grass (7). Both O-GCs and I-GCs have been shown to decrease airway hyperreactivity and inflammation in cats sensitized to A. suum (6). No comprehensive evaluation has been done of the effects of treatment with I-GCs or O-GCs on the major aspects of naturally occurring asthma in cats, namely airway inflammation, anatomical/ radiographical abnormalities, and lung function.

Glucocorticoid therapy, in any form, has the potential to alter the HPA axis, as well as to predispose a cat to the development of diabetes mellitus (DM). While it has previously been demonstrated that inhaled fluticasone suppresses HPA axis activity, the effect of fluticasone on glucose homeostasis, as assessed by fructosamine concentrations, has not been evaluated (6,7). Elevated fructosamine is considered to be an early marker for DM, and while elevations in fructosamine have been identified in healthy cats administered oral prednisolone (15,16), the effect of fluticasone on fructosamine is unknown. Finally, the specific inciting factor for allergic asthma is not often identified clinically. One pilot study (17) showed a marked increase in positive results for intradermal skin and serum testing in cats with asthma, which shows that specific inhalant allergies play a functional role in the development of the asthmatic phenotype.

The objective of this study was to compare the relative ability of inhaled fluticasone with oral prednisolone to resolve airway eosinophilia and radiographic/clinical signs and to decrease airway resistance in naturally occurring feline asthma. The secondary goals were to report the effects of inhaled glucocorticoids (I-GCs) on glucose homeostasis and the results of serum allergy testing in cats with asthma.

Materials and methods

Study cats

Cats with naturally occurring asthma that had not previously been treated were prospectively recruited from the emergency and internal medicine services at the Foster Hospital for Small Animals at the Cummings School of Veterinary Medicine, Tufts University. Asthma was identified based on consistent signalment (> 1 and <10 y old), clinical signs (cough, wheeze, and/or respiratory distress), a generalized bronchial or bronchointerstitial pattern on thoracic radiographs, and airway eosinophilia (≥ 17%) on blind bronchoalveolar lavage (BAL) (18). Cats were excluded from participation if there was evidence of other underlying disease, such as heartworm or lungworm infestation, heart failure, other cardiopulmonary disease, or lack of sufficient airway eosinophilia. The study was approved by the clinical science review committee and the owners provided written informed consent before cats were enrolled in the study.

Study design

Cats were assigned to 1 of 2 groups using a randomization chart: i) O-GC group with administration of 5 mg of prednisolone by mouth twice daily for 14 d and then 5 mg of prednisolone once daily for 6 wk; or ii) I-GC group with administration of 5 mg of prednisolone once daily for the first 7 d and 110 μg of I-GC as fluticasone (Flovent HFA; GlaxoSmithKline, Research Triangle Park,
North Carolina, USA) administered twice daily for the duration of the study using a specially designed spacer and facemask (Aerokat Feline Aerosol Chamber; Trudell Medical, London, Ontario). Owners of cats randomized to the I-GC group were trained on use of the device. Prednisolone was provided to both groups as a compounded chewable tablet (Wedgewood Compounding Pharmacy, Swedesboro, New Jersey, USA). Cats were evaluated at baseline and then again after 8 wk of therapy. Each evaluation included thoracic radiographs, lung function testing, blind BAL, and serum fructosamine.

**Thoracic radiography.** Computed radiographic (CR) images of the thorax from both time points were exported as DICOM images, anonymized to remove patient ID, name, and signalment, and then randomized by a single author (MVK) using an open source anonymizer (DICOM Cleaner; PixelMed Publishing, Bangor, Pennsylvania, USA). All studies were subsequently evaluated by a single observer (TJO), who was Board-certified by the American College of Veterinary Radiology and unaware of the radiograph acquisition time point. Images were viewed using a free and open source DICOM viewer (OsiriX Lite; PixmeoSARL, Geneva, Switzerland). The observer was able to manipulate the images by adjusting window level/width, zoom, and pan. The availability of left lateral, right lateral, dorso-ventral, and ventrodorsal projections was documented.

Radiographs were given a score of 0 to 9 based on the severity of bronchial, interstitial, and alveolar patterns, with each pattern separately graded on a scale of 0 to 3, similar to methods described in previous studies (19,20). Bronchial patterns were graded as: (0) absent; (1) mild, with visualization of first generation bronchi; (2) moderate, with visualization of second generation bronchi; and (3) severe, with visualization of third generation bronchi. Unstructured interstitial patterns were graded as: (0) absent; (1) mild, with generalized slight increase in soft tissue opacity of lung parenchyma that did not blur the vascular margins; (2) moderate, with generalized or focal increased soft tissue opacity of the lung parenchyma that blurred some vascular margins; or (3) severe, with generalized increased soft tissue opacity of lung parenchyma that blurred all vascular margins in the affected lung lobe.

Structured interstitial patterns with any size of soft tissue opaque pulmonary nodules were not present in any cases and were therefore not graded. Alveolar patterns were graded as: (0) absent; (1) mild, with focal “fluffy” mild increase in soft tissue opacity of pulmonary parenchyma without visualization of vascular margins; (2) moderate, as presence of air bronchograms (unilateral or bilateral); or (3) severe, as presence of lobar signs (unilateral or bilateral). All grading was done in a single viewing session. Scores before and after therapy, for presence of lobar signs (unilateral or bilateral). All grading was done in a single viewing session. Scores before and after therapy, for presence of lobar signs (unilateral or bilateral). All grading was done in a single viewing session. Scores before and after therapy, for presence of lobar signs (unilateral or bilateral). All grading was done in a single viewing session. Scores before and after therapy, for presence of lobar signs (unilateral or bilateral). All grading was done in a single viewing session. Scores before and after therapy, for presence of lobar signs (unilateral or bilateral).

**Bronchoalveolar lavage (BAL).** After lung function was determined, blind BAL was carried out using 2 mL/kg BW of warmed saline, repeated 3 times, for a total of 6 mL/kg BW of lavage. All cats received 1 puff of albuterol (90 µg) after lung function was determined and before BAL. The lavage fluid was placed into tubes containing ethylenediamine tetraacetic acid (EDTA). Direct smears and cytocentrifuged preparations of the fluid were made. All slides were stained with a 2-part aqueous Romanowsky stain (Protocol Hemaspray; Fisher Health Care, Hampton, New Hampshire, USA) and an automated slide stainer (Aerospray Hematology Stat Slide Stainer 7122; EliTech Group, Puteaux, France) before being reviewed by 1 of 2 clinical pathologists, both Board-certified by the American College of Veterinary Pathology (JK, PB). Clinical pathologists were unaware of the cat’s treatment status. The total nucleated cell count and the percentage of eosinophils were recorded.

**Systemic effects.** Serum fructosamine was evaluated at baseline and at the completion of the study.

**Serum allergy testing.** A single serum sample was collected at baseline and submitted to a reference laboratory (Allercept Serum Allergen; Heska, Loveland, Colorado, USA) to be tested for evidence of increased reactivity to common inhalant allergies. These included weeds (yellow dock, English plantain, lamb’s quarters, rough pigweed, Russian thistle, short ragweed, burweed marsh elder, rough marsh elder, kochia, tall ragweed, mugwort, and common cocklebur); trees (bayberry wax myrtle, white ash, shagbark hickory, box elder, Eastern cottonwood, American elm, black birch, sugar maple, red mulberry, white oak, American sycamore, black walnut, yellow pine, quaking aspen, and red cedar); grasses (timothy grass, orchard grass, Johnson grass, sweet vernal, meadow fescue, smooth brome, perennial rye grass, red top grass, June bluegrass, and Bermuda grass); fungi (Fusarium roseum, Penicillium notatum/chryosporum, Aspergillus fumigatus, Cladosporium sphaerospermum, and Alternaria tenuis); and environment (Dermatophagoides pterinyssinus, Tyrophagus putrescentiae, flea saliva, cat epithelium, Dermatophagoides farinae, and cockroach).

**Statistical analysis**

Descriptive and summary statistics were calculated. Pre- and post-treatment values for individual patients as well as differences between groups were compared using a Wilcoxon rank-sum test or Fisher’s exact test with a P-value < 0.05 considered significant.

**Results**

A total of 14 cats were enrolled and 9 cats successfully completed the 8-week study. Two cats were excluded after enrollment due to lack of sufficient airway eosinophilia (2% and 5%, respectively). One owner moved despite agreeing to participate in the study and the cat was lost to follow-up and 2 owners failed to administer medications as prescribed, despite verbal and written instructions. Of the 9 cats that completed the study, 8 were domestic short hairs and 1 was a domestic long hair. Four were spayed females and 5 were neutered males. The median age of cats was 2 y (range: 18 mo to 9 y) and the mean weight was 5 ± 1.1 kg. All cats were heartworm antigen and antibody negative, with no evidence of parasites on Baermann fecal sedimentation and fecal flotation. No cats had evidence of external parasites such as fleas.
Four cats were randomized to receive oral glucocorticoid (O-GC) therapy alone and 5 cats to receive inhaled glucocorticoids (I-GCs). All cats responded well to therapy and both owners and attending clinicians considered them clinically normal after 8 wk of treatment. No cats had labored breathing at any point in the study and owners reported that coughing had not been observed in the cats. Both forms of therapy were well-tolerated by cats and owners, and no owners reported difficulty giving the medications. Further information about clinical signs at home was not recorded.

**Thoracic radiographs**

Radiographs were available for review in 8 cats both before and after treatment. In 1 cat in the O-GC group, only post-treatment radiographs were available for review. In this cat (number 6), the post-treatment score was 4. In the 8 cats with pre- and post-treatment radiographs, the median score decreased insignificantly from 4 to 2 \((P = 0.11)\). In the O-GC group \((n = 3)\), the median pre-treatment score was 4 and the median post-treatment score was 3. Two cats had a decrease in score and 1 cat had an increase in score from pre- to post-treatment. In the I-GC group \((n = 5)\), the median pre-treatment score was 4 \((range: 1 \text{ to } 5)\) and the post-treatment score was 2 \((range: 1 \text{ to } 3)\). Four of the 5 cats had a decrease in score and 1 cat increased from 1 to 3. There was no difference in the number of cats that improved radiographically or the degree of change between groups.

**Airway eosinophilia**

In accordance with the study design, all cats had airway eosinophilia at the time of enrollment. Airway eosinophilia decreased significantly in all cats \((P = 0.003)\) from a median of 51% eosinophils to a median of 9%. There was a decrease of 48% to 6% in the O-GC group and a decrease of 51% to 19% in the I-GC group (Table I). There was no difference in reduction between groups as there was significant interindividual variation in response. Using 17% eosinophils as the therapeutic target point \(\text{based on normal eosinophil percentages proposed in previous articles (21,22)}\), 3 out of 4 cats \(75\%\) in the O-GC group, compared to 2 out of 5 cats in the I-GC group, achieved this level of improvement \((P = 0.52)\). Total nucleated cell count decreased significantly from a median of 4960/\(\mu\)L \((range: 2880 \text{ to } 21300/\mu\)L) to 2810/\(\mu\)L \((range: 810 \text{ to } 5720/\mu\)L) with a \(P = 0.03\).

**Lung function testing.** Results of lung function tests in individual cats are shown in Table I. The median lung resistance \((R\text{stat})\) was 48.3 cmH\(_2\)O/L/s \((range: 36.6 \text{ to } 109.7 \text{ cmH}_2\text{O}/\text{L/s})\). The baseline median Rstat was higher in the O-GC group at 89.7 cmH\(_2\)O/L/s than in the I-GC group at 42 cmH\(_2\)O/L/s \((P = 0.032)\).

After treatment, the median Rstat for all cats decreased from 48.3 cmH\(_2\)O/L/s to 35 cmH\(_2\)O/L/s \((P = 0.02)\). Within groups, the median Rstat in the O-GC group was lower after treatment at 35 cmH\(_2\)O/L/s \((P = 0.032)\) compared to 89.7 cmH\(_2\)O/L/s at baseline \((P = 0.03)\), while in the I-GC group, the median Rstat after treatment was 35 cmH\(_2\)O/L/s, which was not statistically different from baseline \((P = 0.54)\).

There was no correlation \((r = 0.27)\) between percentage of eosinophils in bronchoalveolar lavage fluid \((\text{BALF})\) and airway resistance. Cats with 50% or more eosinophils had a median Rstat of 57 cmH\(_2\)O/L/s \((range: 42 \text{ to } 7420/\mu\)L) while in the I-GC group, the median Rstat after treatment was 35 cmH\(_2\)O/L/s \((P = 0.86)\). No cat developed hyperglycemia or any signs of polyuria/polydipsia/polyphagia.

**Fructosamine**

There was no change in median fructosamine with treatment; median level before treatment was 217 \(\mu\)mol/L \text{ versus } 227 \(\mu\)mol/L after treatment \((P = 0.86)\). No cat developed hyperglycemia or any signs of polyuria/polydipsia/polyphagia.

**Serum allergy testing**

Serum allergy results were available for 8 cats. Four cats had no detectable allergen-specific IgE and 4 cats had evidence of detectable serum-specific IgE (Table I).

---

### Table I. Baseline characteristics of enrolled cats and response to therapy.

<table>
<thead>
<tr>
<th>Cat number</th>
<th>Inhaled glucocorticoid group</th>
<th>Oral glucocorticoid group</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAL eosinophil % and total nucleated cell count/(\mu)L</td>
<td>Static resistance ((R\text{stat}; \text{cmH}_2\text{O}/\text{L/s}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-treatment</td>
<td>Baseline</td>
<td>Post-treatment</td>
</tr>
<tr>
<td>1</td>
<td>58; 4880</td>
<td>43; 7520</td>
<td>57.7</td>
<td>56.6</td>
</tr>
<tr>
<td>2</td>
<td>48; 9450</td>
<td>19; 3800</td>
<td>36.6</td>
<td>32.67</td>
</tr>
<tr>
<td>3</td>
<td>84; 21300</td>
<td>7; 1760</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>51; 7880</td>
<td>44; 834</td>
<td>39.3</td>
<td>33.4</td>
</tr>
<tr>
<td>5</td>
<td>42; 7420</td>
<td>1; 3370</td>
<td>42.33</td>
<td>81</td>
</tr>
</tbody>
</table>

*BALF — bronchoalveolar lavage; immunoglobulin E-specific allergen is classified as either positive or negative; \(F.\) roseum — \(Fusarium\) roseum; \(T.\) putrescentiae — \(Tyrophagus\) putrescentiae (dust mite); \(D.\) farina — \(Dermatophagoides\) farinae.*
**Discussion**

In this small 8-week pilot clinical trial, treatments with both inhaled and oral glucocorticoids (I-GCs and O-GCs) were effective in eliminating clinical signs and reducing airway eosinophilia in cats with naturally occurring asthma. Both O-GCs and I-GCs were well-tolerated by all treated cats. Oral glucocorticoids (O-GCs) were associated with a more robust improvement in airway resistance than inhaled GCs in cats as measured using a critical care ventilator, although the baseline resistance was significantly higher in cats that were randomized to the O-GC group.

Interestingly, while all cats were clinically normal after therapy, there was persistent evidence of airway inflammation (eosinophilia) in 4 cats and evidence of increased airway resistance (> 50 cmH₂O/L/s) in 2 cats. This suggests that assessing response to therapy may require re-sampling of the airways, rather than just evaluating clinical signs and radiographs in order to truly resolve all changes associated with feline asthma.

In a similar study in horses comparing inhaled glucocorticoids and oral dexamethasone to treat equine asthma, both delivery methods improved airway function, with variable results on airway cytology (23). It has been demonstrated that fluticasone is more effective in long-term prevention and treatment of equine asthma than in acute improvement in a crisis (24) and the same may be true in cats. In human medicine, inhaled corticosteroids are the recommended first choice for treating asthma, although oral corticosteroids are acceptable if inhaled options are not available or in severe/refractory cases (8).

The underlying reason for development of spontaneous feline asthma is unknown. Since exposure to inhaled allergens, e.g., Bermuda grasses, has been effective in creating a model of feline asthma, immunotherapy has been proposed as a method of treating cats with spontaneous feline asthma. Interestingly, only half of the cats in this study showed evidence of serum IgE-specific reactivity. This is similar to a previous study (17) in which 34% of allergens tested were positive in cats with asthma. The cats that demonstrate serum IgE-specific reactivity may represent a cohort of cats that would respond to desensitization therapy, which was not evaluated in this study. Intradermal skin testing, which may be more specific for a true allergic phenotype, was not done in these cohorts and this may have led to a misrepresentation of cats with identified allergies.

This study evaluated airway eosinophilia as an outcome. The day-to-day repeatability of blinded BAL in cats is unknown, whereas it is considered to be reproducible in humans (25). This study used 17% eosinophils as a cutoff based on previous work (3) and used in prior studies (18,22). A more recent paper used 5% eosinophils as a cutoff for healthy cats (26). Using 5% as a cutoff, only 1 of 5 cats reached that target with I-GC and 2/4 cats with O-GC (P = 0.52). A more stringent cutoff for airway eosinophilia might therefore be considered in future studies. Airway eosinophilia has been identified as being related to enhanced pause (PENH), which is an indirect measure of airway resistance, in cats with chronic bronchitis that are challenged with carbachol (6). Similar results were not found in this small study, although cats with higher airway eosinophilia did have a nonsignificant increase in airway resistance.

This study used a critical care ventilator to characterize changes in airway resistance in cats with naturally occurring asthma. Airway resistance primarily reflects narrowing of the larger airways, with higher resistance associated with more airway narrowing. Similar to humans and horses, cats develop naturally occurring bronchoconstriction, which should lead to higher airway resistances. While testing of pulmonary function has been used extensively in human medicine and in research settings to characterize airway disease, there has been limited crossover to clinical veterinary patients, including cats (14).

In the Bernhard study, experimentally induced asthmatic cats had a baseline airway resistance as measured by ventilator mechanics of 55.2 ± 7.8 cmH₂O/L/s (14). This was similar to the median of 48.3 cmH₂O/L/s observed in the present study. Using dedicated equipment for testing pulmonary function, Dye et al (2) identified airway resistance of 38 to 45 cmH₂O/L/s in mild to moderately affected cats with naturally occurring asthma. This supports the use of critical care ventilator mechanics as a reasonable surrogate for more dedicated equipment for testing pulmonary function. As more veterinary practices acquire critical care ventilators, the opportunity to routinely test lung function in practice is increasingly common and may be easily combined with collection of BALF (27). One significant limitation of this study was using baseline lung function testing, rather than following aerosol challenge with histamine or carbachol, as airway hyperresponsiveness is a key feature of asthma (6,28). We chose not to conduct bronchoprovocation in client-owned animals due to its associated risks.

There was no significant effect of treatment on the severity of radiographic scores for cats enrolled in either treatment arm of this study, which is similar to previous results in experimental models (6). The visible radiographic changes, i.e., bronchial pattern, associated with feline lower airway disease are the result of thickened bronchi/conducting airways. This thickening likely represents a summation of effects from smooth muscle hypertrophy, eosinophilic infiltrate, and mucus production resulting from chronic inflammation. While treatment will decrease mucus production and cellular infiltrates, it is not likely to fully reverse the airway remodeling, i.e., muscle hypertrophy, that occurs with this condition. Furthermore, given the lack of correlation between measured airway resistance and radiographic score, thoracic radiographs are probably not the most effective surrogate for assessing treatment efficacy, despite their wide availability.

In addition to potential limitations due to underlying non-reversible airway changes, other limitations of thoracic radiograph evaluation in this study included the use of a single observer, as well as differences in radiographic projections available for review, i.e., orthogonal versus single lateral, and radiographic technique, i.e., patient motion, rotation, or pulmonary underinflation.

The dose of I-GC administered in this study was chosen based on previous studies that used cats with induced airway disease (5–7). Based on the limited absorption due to conformation, some clinicians feel that a higher dose, e.g., 220 µg/puff, would be more effective. The ideal dose of inhaled glucocorticoids for cats remains to be determined. In a similar study using an asthma model, a higher dose of fluticasone (500 µg twice daily) combined with 50 µg of salmeterol was effective in decreasing airway eosinophilia and hyperresponsiveness (6).

Similarly, the dose of prednisolone chosen for the O-GC group was 5 mg every 12 to 24 h and it is possible that a higher dose
would be more effective. This study chose not to compound based on individual cat weights in order to more closely mimic a typical clinical approach.

One limitation of this study was the substantial variability in results of BAL, radiographs, and lung function testing for cats with clinical signs consistent with feline asthma. This natural variability may influence the severity of disease, as well as the response to therapy, and may reflect the difference in outcome in individual cats in this pilot study rather than a specific difference in treatment efficacy.

Another limitation included lack of Mycoplasma culture or polymerase chain reaction (PCR) in all cats. It is recognized that Mycoplasma infection or co-infection may be documented in cats with airway disease (29), although the role of Mycoplasma in feline respiratory disease is not known. Bordetella bronchiseptica has also been isolated in cats with cough (2,30). Additionally, despite randomization of the groups, the O-GC group had a significantly higher baseline resistance, suggesting that the underlying severity of disease might have been different from the I-GC group. Finally, it is possible that owners of cats in either group were less effective at administering the medication than they reported.

The most significant limitation of this pilot study was sample size; only 9 cats were ultimately enrolled and completed the study, with an additional 5 cats being enrolled, but not completing the study. A post-hoc analysis of blind BAL eosinophilia showed that 60 cats (30 per group) would have been required to demonstrate a significant difference between groups.

In conclusion, both oral and inhaled glucocorticoids (O-GCs and I-GCs) improve lung function and reduce airway eosinophilia. This pilot study was underpowered to detect a difference in therapeutic efficacy. Persistent airway inflammation after treatment was common, despite the apparent lack of clinical signs. Repeated BAL and/or pulmonary function testing may be necessary to document treatment efficacy for asthma as thoracic radiograph changes did not resolve with therapy. Further investigation is required to better elucidate the underlying causes of feline asthma, as well as to determine the most appropriate treatment options.

Acknowledgment

This study was funded by a grant from Trudell Medical International in London, Ontario.

References


Errata

The corresponding author has requested that the following changes be made to his article which appeared in the October 2020 issue (CJVR 2020;84:272–282).

Abstract

The objective of this study was to compare the efficacy of commercially available porcine circovirus type 2 (PCV2) and Mycoplasma hyopneumoniae vaccines. A total of 80 pigs was randomly divided into 6 treatment groups; 4 of the groups each received a different vaccine as well as a dual challenge. The remaining 2 groups were used as controls, 1 of which also received a dual challenge. Two of the 4 groups of pigs were administered 2 monovalent vaccines (designated as either monovalent vaccine A or B) of PCV2 at 7 days old and of M. hyopneumoniae at 21 days old. The remaining 2 vaccinated groups of pigs received a bivalent vaccine (designated as either bivalent vaccine A or B) of PCV2 and M. hyopneumoniae at 21 days old. All 4 vaccinated groups were challenged with M. hyopneumoniae at 42 days old (14 days post-challenge [dpc]), followed by a PCV2 challenge at 14 dpc.

Corrections:

Abstract

2 monovalent vaccines (designated as either monovalent vaccine A or B) of M. hyopneumoniae at 7 days old and PCV2 at 21 days old, or M. hyopneumoniae and PCV2 at 21 days old.

Résumé
deux vaccins monovalents (identifiés comme vaccin monovalent A ou B) de M. hyopneumoniae à 7 jours d’âge et PCV2 à 21 jours d’âge, ou M. hyopneumoniae et PCV2 à 21 jours d’âge.

Figure 1. Experimental design. Pigs were administered a vaccine against M. hyopneumoniae (Mhp) and/or porcine circovirus type 2 (PCV2) and challenged with M. hyopneumoniae and PCV2 on certain days as shown. A number of pigs were necropsied as shown.
Analysis of gene expression of prostaglandin EP4 receptor in canine osteosarcoma

Margaret L. Musser, Austin K. Viall, Rachel L. Phillips, Jesse M. Hostetter, Chad M. Johannes

Abstract

In many human cancers, the expression of the prostaglandin receptor EP4 (EP4R) is associated with the development of malignancy and a poor prognosis. The expression of EP4R has not yet been evaluated in canine tumors. The objective of this study was to characterize the messenger RNA (mRNA) expression of EP4R in canine osteosarcoma (OSA). Gene expression of EP4R was evaluated using RNA in-situ hybridization (RNAscope). In all canine OSA samples evaluated, strong universal positive expression of EP4R was identified. Gene expression was significantly higher in OSA tissue samples than in normal nasal turbinate bone, possibly implicating EP4R in the pathogenesis of canine OSA.

Résumé


Osteosarcoma (OSA) is diagnosed in 85% of canine malignant skeletal tumors, making OSA the most common canine primary bone tumor (1). Osteosarcoma is an aggressive neoplasm, with destructive local bone involvement and over 90% of dogs developing distant metastatic disease within 1 y of diagnosis (1). Standard of care includes amputation or limb-sparing procedure (surgical or radiation therapy-based), followed by adjuvant chemotherapy. Amputation alone typically results in a survival time of 5.4 mo (1). Several adjuvant chemotherapy protocols have been assessed, resulting in a large range of median survival times (7 to 18 mo) (1). Despite aggressive therapy, most dogs with OSA succumb to metastatic disease, which indicates that alternative treatment modalities are needed to improve overall outcome.

Inflammation and immune activation triggered by a carcinogenic stimulant can promote the development of cancer (2). This is typically mediated through upregulation of non-specific pro-inflammatory cytokines and enzymes, the most important of which is the cyclooxygenase enzyme 2 (COX-2). This enzyme stimulates angiogenesis, inhibits apoptosis, and promotes cell proliferation and motility through production of prostaglandin E2 (PGE2) (2). The activities of PGE2 are mediated through its 4 recognized receptors [EP1 receptor to EP4 receptor (EP1R to EP4R)] on the surface of the target cells (3).

Canine OSA has been shown to overexpress COX-2, suggesting that COX-2 is a reasonable therapeutic target (4). Blocking COX-2, however, decreases the production of complementary prostanoids that are important in homeostasis and could result in significant side effects (5). Treatment options that target the prostaglandin pathway more specifically may therefore be advantageous.

Many malignancies in humans and dogs have been shown to overexpress COX-2 and/or PGE2 (6,7). In addition, the EP receptors, EP4R in particular, are expressed and associated with the development of malignancy and poor prognosis in several human cancers (8). Although characterization of the canine EP4R has been completed (9) and expression of EP2R has been confirmed in canine OSA (4), it is unknown if EP4R is overexpressed in canine OSA.

Based on the available human and canine literature, it is reasonable to hypothesize that expression of EP4R may be present in canine OSA and may play a role in its development and progression. The objective of this study was to evaluate the gene expression of EP4R in canine OSA using the novel in-situ hybridization platform, RNAscope.

Archived biopsy and necropsy tissue specimens maintained by the Department of Veterinary Pathology at Iowa State University were searched for tissue samples of formalin-fixed, paraffin-embedded appendicular OSA and normal nasal turbinate bone. The aim was
Table 1. Characterization of evaluated appendicular osteosarcoma samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Breed of dog</th>
<th>Anatomic location</th>
<th>Histologic diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Doberman pinscher</td>
<td>Left distal radius</td>
<td>Osteoblastic osteosarcoma</td>
</tr>
<tr>
<td>2</td>
<td>Labrador retriever</td>
<td>Right proximal femur</td>
<td>Osteoblastic osteosarcoma</td>
</tr>
<tr>
<td>3</td>
<td>Labrador retriever</td>
<td>Left proximal tibia</td>
<td>Osteoblastic osteosarcoma</td>
</tr>
<tr>
<td>4</td>
<td>Australian shepherd</td>
<td>Left proximal humerus</td>
<td>Osteoblastic osteosarcoma</td>
</tr>
<tr>
<td>5</td>
<td>Mixed breed</td>
<td>Left distal radius</td>
<td>Osteoblastic osteosarcoma</td>
</tr>
<tr>
<td>6</td>
<td>Rat terrier</td>
<td>Left distal femur</td>
<td>Osteoblastic osteosarcoma</td>
</tr>
<tr>
<td>7</td>
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<td>Fibroblastic osteosarcoma</td>
</tr>
<tr>
<td>8</td>
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<td>Right proximal humerus</td>
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<td>9</td>
<td>Shetland sheepdog</td>
<td>Left proximal humerus</td>
<td>Chondroblastic osteosarcoma</td>
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Figure 1. EP4R mRNA expression metrics in canine osteosarcoma and normal nasal turbinate bone. Data presented as mean +/- 95% confidence interval (CI) of mean and individual data points.

* Denotes differences were statistically significant.

to collect 9 specimens each of appendicular OSA and normal bone with adequate RNA for analysis. Based on preliminary data, a power calculation indicated that 9 specimens in each group would be statistically adequate to identify a difference of 2 transcript copy numbers/cell between tumor and normal bone (alpha = 0.05, beta = 0.2). The Veterinary Teaching Hospital and Department of Veterinary Pathology allowed the authors to use the clinical data and samples from specimens gathered through routine clinical assessment and care.

The RNAscope mRNA in-situ hybridization platform (Advanced Cell Diagnostics, Newark, California, USA) was used to evaluate messenger RNA (mRNA) expression of EP4R gene ptger4 in the appendicular OSA and normal bone tissue samples. According to the manufacturer’s instructions and as previously reported (10), single-plex, manual chromogenic RNAscope analysis was conducted with an RNAscope 2.5 High Definition (HD)-RED Assay (Catalog #322350; Advance Cell Diagnostics). Briefly, 3 sections of each paraffin-embedded OSA specimen were cut to a 5-μm depth. Preparations were baked for 1 h at 60°C, deparaffinized, and protease treated to expose RNA. The 3 sections were then hybridized separately with a test probe targeting canine ptger4 (Probe CI-PTGER4, Catalog #499011; Advanced Cell Diagnostics), a positive control probe targeting canine housekeeping gene *ube* (CI-UBC Positive Control, Catalog #409851; Advanced Cell Diagnostics), and a negative control probe targeting *Bacillus subtilis* dapB (DapB Negative Control, Catalog #310043; Advance Cell Diagnostics).

Hybridization to target mRNA was carried out by incubating the preparation with the respective probe at 40°C for 2 h in a HybEZ hybridization oven (Advanced Cell Diagnostics). Subsequent wash and signal amplification steps were taken according to the manufacturer’s protocol. Target mRNA was detected using alkaline phosphatase Fast Red chromogenic stain (Catalog #320701; Advanced Cell Diagnostics). Samples were also stained with hematoxylin (American Master Tech Scientific, Lodi, California, USA) to allow visualization of nuclei. To initially assess the performance of the ptger4 RNAscope experimental probe, ptger4 expression was assessed in 2 sections each of normal canine heart, lung, and kidney tissue. Expression of ptger4 has previously been reported in these canine tissues (9) and was identified in all 3 tissue types with the RNAscope analysis with this ptger4 probe.

For each hybridized slide, 10 400× magnified non-overlapping microscopic field views were digitally photographed. Digitized photomicrographs were then evaluated with the RNAscope image analysis software HALO with the RNAscope Modules (Indica Labs, Albuquerque, New Mexico, USA) (11). For tumor samples, the neoplastic cells were manually identified post-processing and gated for EP4R analysis. To ensure neoplastic cells were appropriately identified for analysis, a hematoxylin and eosin histological correlate of the exact tissue block was simultaneously evaluated. For normal tissue samples, the appropriate normal cell population was manually identified and gated for analysis. Samples were considered to have adequate residual RNA for ptger4 expression.
The mean copy number per cell [OSA 1.10, 95% CI: 0.53 to 1.74; nasal turbinate 0.18, 95% CI: 0.11 to 0.24; P-value 0.0022]; H-score (OSA 37.4, 95% CI: 20.9 to 53.6; nasal turbinate 6.1, 95% CI: 3.9 to 8.3; P-value 0.0001); and percent probe positive scores (OSA 22.6, 95% CI: 14.5 to 30.8; nasal turbinate 3.8, 95% CI: 2.3 to 5.4; P-value 0.0001) were statistically higher in the OSA samples (specifically the malignant OSA cells) than in the normal nasal turbinate samples (Figures 1 and 2).

It was found that the canine OSA tumor cells had significantly higher gene expression of EP4R than the normal turbinate cells. Although the true cell of origin in the development of OSA is still debatable, recent literature suggests that OSA most likely forms from mutated osteoblasts or cells that are committing to being of osteoblast lineage (12). The 2 most important EP receptors on normal osteoblasts are EP2 and EP4 (13). Notably, in a recent model of periodontal disease, the human OSA cell line Saos-2 was used to evaluate the impact of lipopolysaccharide (LPS) on the production of PGE2 and expression of EP4R. It was found that exposure to LPS significantly increased PGE2 production and subsequent gene expression of EP4R (14).

Prostaglandin signaling through EP4R stimulates monocytes to form mature osteoclasts, leading to the classic osteolysis seen in metastatic bone lesions (15). Specifically, direct cell-cell contact between the tumor cells and osteoblasts activates the NFkB/mitogen-activated protein (MAP) kinase pathway, increasing expression of COX-2. Subsequently, increased COX-2 leads to additional PGE2 secretion, which binds to EP4R on nearby osteoblasts, increasing

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**Figure 2.** Analysis of *ptger4* transcription in a section of nasal turbinate bone (A, B, C) and osteosarcoma (D, E) following RNAscope mRNA in-situ hybridization with hematoxylin counterstain. A — Native photomicrograph to be analyzed for copy numbers/cell, H-score, and percentage transcript expression using HALO software with RNAscope modules. B — Nasal turbinate bone was manually gated (blue line) for analysis. C — HALO-generated probe markup from which copy numbers/cell, H-score, and percentage transcript expression are calculated. D — Native photomicrograph to be analyzed for copy numbers/cell, H-score, and percentage transcript expression using HALO software with RNAscope modules. E — HALO-generated probe markup from which copy numbers/cell, H-score, and percentage transcript expression are calculated; no blue gate line applied since entire field consisted of neoplastic cells.
production of receptor activator for NFkB ligand (RANKL), induc-
ing osteoclastogenesis via receptor activator for NFkB (RANK) (15). Inherently increased EP4R expression would likely promote this process. Expression of RANKL has been identified in canine OSA (16), the presence of which could theoretically be attributed to increased EP4R, which would make EP4R an attractive therapeutic target.

The expression of EP receptors is associated with the development of malignancy and poor prognosis in several human cancers (8). Positive gene expression of EP4R mRNA in canine OSA samples suggests that EP4R expression may play a role in the pathogenesis and development of canine OSA.

While this is exciting preliminary data, it must be interpreted in light of the small sample size and the inherent differences between appendicular bones, which are normally affected by OSA, and nasal turbinate bone, which was used as the control tissue. The impact of prolonged decalcification on the interpretation of RNAscope is not known. Anecdotally, it does not appear to affect results, but out of an abundance of caution, it was elected to use nasal turbinate bone that requires less decalcification, potentially having less of an impact on the results. In addition, this work is limited in scope and does not confirm the protein expression of EP4R. Similarly, the prognostic value of increased EP4R expression and its potential as a viable therapeutic target was not fully evaluated. As this represents a pilot and proof-of-concept study, additional tumor specimens, along with appendicular bone and reactive bone, the protein expression of EP4R, and its value as a therapeutic target will need to be evaluated to confirm the results of this study.

Acknowledgments

The authors thank Dr. Olufemi Fasina for his assistance with the histopathological characterization of the osteosarcoma (OSA) samples. This research was generously supported by Fetch-a-Cure and internal funding opportunities from Iowa State University.

References

Changes in mRNA of immune factors expressed by milk somatic cells of Holstein cows with hypocalcemia after calving

Hiromichi Ohtsuka, Misa Ohsawa, Kenji Murakami, Ryo Murata, Toshihide Kato, Motoshi Tajima

Abstract

Changes in immune factors expressed by milk somatic cells from Holstein cows with hypocalcemia after calving were investigated in this study. Fourteen multiparous Holstein cows after their 3rd or 4th calving in one farm were used. The cows were divided into 2 groups: 7 cows needing treatment due to onset of hypocalcemia (hypocalcemia group; age = 5.53 ± 0.27 years, parity = 3.14 ± 0.14) and 7 cows without health problems (control group; age = 5.88 ± 0.31 years, parity = 3.57 ± 0.26). Milk samples were collected aseptically using a cannula and mRNA of immune factors expressed by milk somatic cells were analyzed. Milk samples (50 mL) were collected from the right rear mammary gland of cows before milking at day 1 and weeks 1, 2, 4, and 8 after calving. All milk samples showed a negative reaction to the California Mastitis Test. Levels of relative interleukin (IL)-6 and cathelicidin in the hypocalcemia group were lower than those in the control group in weeks 1 to 8. A significant difference in relative IL-6 levels was found in week 4 (P < 0.05). These results suggest that levels of IL-6 expressed by milk somatic cells may be affected by hypocalcemia in dairy cows.

Résumé

Dans la présente étude les modifications des facteurs immunitaires exprimés par les cellules somatiques du lait de vaches Holstein présentant une hypocalcémie après le vêlage ont été examinées. Quatorze vaches Holstein multipaires après leur 3e ou 4e vêlage provenant d’une ferme ont été utilisées. Les vaches ont été réparties en deux groupes : sept vaches nécessitant un traitement en raison de l’apparition d’une hypocalcémie (groupe hypocalcémie; âge = 5,53 ± 0,27 ans, parité = 3,14 ± 0,14) et sept vaches sans problème de santé (groupe témoin; âge = 5,88 ± 0,31 ans, parité = 3,57 ± 0,26). Des échantillons de lait ont été prélevés de manière aseptique à l’aide d’une canule et l’ARNm des facteurs immunitaires exprimés par les cellules somatiques du lait a été analysé. Des échantillons de lait (50 mL) ont été prélevés dans la glande mammaire arrière droite des vaches avant la traite au jour 1 et aux semaines 1, 2, 4 et 8 après le vêlage. Tous les échantillons de lait ont montré une réaction négative au California Mastitis Test. Les niveaux relatifs d’interleukine (IL)-6 et de cathélidine dans le groupe hypocalcémie étaient inférieurs à ceux du groupe témoin au cours des semaines 1 à 8. Une différence significative des taux relatifs d’IL-6 a été observée à la semaine 4 (P < 0.05). Ces résultats suggèrent que les taux d’IL-6 exprimés par les cellules somatiques du lait peuvent être affectés par l’hypocalcémie chez les vaches laitières.

School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan (Ohtsuka, Ohsawa, Murata, Kato, Tajima); Federation of Agricultural Mutual Relief Association, Fukagawa, Hokkaido 073-0022, Japan (Murakami).

Address all correspondence to Dr. Hiromichi Ohtsuka; telephone: +81-11-388-4787; fax: +81-11-386-1214; e-mail: ohtsuka@rakuno.ac.jp

Received March 6, 2020. Accepted July 3, 2020.
Fourteen multiparous Holstein cows (immediately after their 3rd or 4th calving) in one farm were divided into 2 groups for this study: 7 cows with onset of hypocalcemia that required treatment (hypocalcemia group; age = 5.53 ± 0.27 y, parity = 3.14 ± 0.14) and 7 cows that remained clinically healthy (control group; age = 5.88 ± 0.31 y, parity = 3.57 ± 0.26). The hypocalcemia group showed clinical signs such as lethargy, inappetence, weak heartbeat, and low body surface temperature. Mean serum calcium was 6.91 ± 0.26 mg/100 mL [mean ± standard error (SE)] on the day of calving. Treatment was conducted 5.29 ± 0.14 d after calving. During the morning milking (by hand), 50 mL of each milk sample was collected aseptically from the mammary gland cistern of the right rear quarter using a cannula, after disinfecting the teat end. Sampling of milk was conducted once a week on weeks 0 (on the day of calving), 1, 2, 4, and 8 after calving. Immediately after sampling, the California Mastitis Test was carried out, and all milk samples showed a negative reaction. These samples were analyzed for mRNA of immune factors expressed by milk somatic cells. The procedures used herein were in accordance with the principles and guideline for animal use set by the Animal Care and Use Committee of Rakuno, Gakuen University in Ebetsu, Japan.

The isolation of total RNA molecules and the real-time PCR of mRNA from activated T-cells; STIM — stromal interaction molecule; STAT — signal transducer and activator of transcription.

PCR — polymerase chain reaction; IL — interleukin; LYZ — lysozyme; CATH — cathelicidin; NFAT — nuclear factor of activated T-cells; STIM — stromal interaction molecule; STAT — signal transducer and activator of transcription.

Milk samples were centrifuged at 400 × g for 10 min at room temperature. After centrifugation, sedimented milk somatic cells were washed twice with PBS and centrifuged at 400 × g for 5 min. For total RNA isolation and cDNA synthesis, a SuperPrep Cell Lysis & RT Kit for qPCR (TOYOBO, Osaka, Japan) was used. Two × 10^6 cells from each sample were mixed with 50 μL of the cell lysis mixture (including gDNA remover) for 5 min before adding the stop solution. Then, 8 μL of each mRNA solution extracted from 32 μL of reaction mixtures was added. cDNA was synthesized using PCR. Real-time PCR was set up using 2 μL first-strand cDNA template, 7.4 μL deionized H₂O, 0.3 μM upstream and downstream primers, and 10 μL SYBR Green Master Mix with ROX reference dye (THUNDERBIRD SYBR qPCR Mix; TOYOBO) on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Waltham, Massachusetts, USA). The following genes were selected: β-actin, IL-1β, IL-6, lysozyme (LYZ), CATH, nuclear factor-kappa B1(NF-κb), NFAT, STIM-1, signal transducer and activator of transcription (STAT)-1, and STAT-3. Primers listed in Table I were designed using the publicly available web-based Primer3 program (12). The Ct values define the threshold cycle of PCR at which amplified products were detected. Real-time PCR reactions were run in duplicate. The final quantification of mRNA of immune-related molecules were carried out using the comparative Ct method. This method was used based

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Figure 1. The time course changes in expressions of interleukin (IL)-1β, IL-6, lysozyme (LYZ), cathelicidin (CATH), nuclear factor of activated T-cells (NFAT), stromal interaction molecule (STIM)-1, signal transducer and activator of transcription (STAT)-1, and STAT-3 after calving. White circles represent the hypocalcemia group and black circles represent the control group. Results are expressed as mean ± standard error (n = 7).

* Significant difference between 2 groups (P < 0.05).
** Significance difference within the same group (P < 0.05).
*** Significance difference within the same group (P < 0.05).
on a validation experiment, which demonstrated that the efficiencies of the target and reference genes were approximately equal, as previously described (13). Expression levels were normalized to β-actin levels and reported as relative fold difference compared to one of the samples arbitrarily chosen as the calibrator. One sample to calibrate was the designated reference, with the lowest amount of mRNA for each target factor gene of all the samples for the respective mRNA.

Statistical analysis for each parameter was performed using a Mann-Whitney U-test to determine the difference between 2 groups at the same sampling time. A Tukey-Kramer test was used to determine the differences among the different sampling weeks within the same group for each parameter. The differences were considered significant at \( P < 0.05 \). Data were expressed as mean ± SE. Correlations between time course changes in parameters were analyzed using Spearman’s rank correlation test and significance was determined at \( P < 0.01 \) or \( P < 0.05 \).

Figure 1 illustrates time course changes in mRNA expression levels of immune factors by milk somatic cells. The level of IL-6 in the control group was lowest in week 0 and increased gradually, reaching its peak in week 4, which was significantly higher compared with week 0 (\( P < 0.05 \)). Significant change in IL-6 level was not found in the hypocalcemia group. The IL-6 level in the hypocalcemia group was significantly lower than in the control group in week 4 (\( P < 0.05 \)). Levels of CATH were lower in the hypocalcemia group than the control group and lasted throughout the experimental period, but the difference between groups was not significant. Changes in NFAT in the control group resembled those of IL-6 and were significantly higher in week 4 compared to week 0 (\( P < 0.05 \)). However, the level of this factor was stable in the hypocalcemia group during the experimental period. The STAT-3 level in the control group was lowest in week 0 and increased after that, showing a significant difference in week 8 (\( P < 0.05 \)). In the hypocalcemia group, STAT-3 was lowest in week 1 and became significantly higher in week 8 compared to week 1 (\( P < 0.05 \)). There was no significant difference in the levels of IL-1β, LYZ, STIM-1, and STAT-1 between the groups and significant changes in these levels within the same group were not detected during the experimental period.

A significant positive correlation was detected between IL-6 and NFAT levels (\( r = 0.648, P < 0.01 \)), as well as CATH and STAT-3 levels (\( r = 0.560 \) and \( r = 0.501 \), respectively, \( P = 0.01 \)). In addition, there was significant correlation of NF-κB with STIM-1 and STAT-1 levels (\( r = 0.902 \) and \( r = 0.912 \), respectively, \( P < 0.01 \)).

This study showed that transcription factors of NFAT, STAT-3, and IL-6 may affect CATH expression rather than the transcription factors of NF-κB (data not shown), STIM-1, and STAT1 in milk somatic cells of cows with hypocalcemia. Cathelicidins have been shown to be one of the pivotal antimicrobial arsenals of leucocytes in the mammary glands of cows. A previous report stated that the CATH peptide levels in milk increased during induced mastitis (14). However, we reported previously that there are significantly lower mRNA levels of IL-6 and CATH in milk containing bacteria compared to milk without bacteria in healthy cows (i.e., without mastitis) (11). Cathelicidin is an antimicrobial peptide with an immune-modulatory function and its expression is induced by IL-6 in immune cells (15). Since we observed significant correlations between IL-6 and CATH levels throughout the experimental period, as well as significantly lower IL-6 in week 2 in the hypocalcemia group, IL-6 may play an important role in the immune system of the mammary gland of cows during the lactation period. Although IL-6 is a well-known inducer of acute inflammatory responses in mastitis (16), this cytokine belongs to the Th2-type cytokine group, which is involved in enhancing and supporting humoral immune responses. A previous report indicated that IL-6 regulates an appropriate immune response by balancing influxes of neutrophils and monocytes (17); therefore, IL-6 may play a pivotal regulatory role in the mammary immune response.

The nuclear factors of activated T-cells, a family of transcription factors expressed both inside and outside of immune cells, are basically calcium-dependent transcription factors, which can be activated by stimulation of receptors coupled to calcium-calmodulin signals (18). Although a significant increase in NFAT expression was found in the control group, this expression was stable and tended to be lower in the hypocalcemia group after week 2. The canonical NFAT activation pathway by Ca\(^{2+}\)/calmodulin signaling has been extensively reviewed and inhibition of NFAT prevents IL-6-driven IL-4 production and Th2 differentiation (19). A previous study, however, implied that the expression of inflammatory mediators that respond early to inflammatory stimulation, such as TNF-α, may be more dependent on NF-κB signaling pathways. Lower IL-6 expression in the hypocalcemia group after week 2 might have been affected by a decline in NFAT signaling pathway activities rather than NF-κB, STIM-1, and STAT-1 signaling pathways.

In healthy cows, expression of IL-6, CATH, NFAT, and STAT-3 by milk somatic cells was at its lowest during the perinatal period and then increased. These changes are considered to be physiological. We initially assumed that immune factors, intracellular transcription factors, or calcium signaling pathway factors would markedly decline in milk somatic cells after calving. But we found a significant difference only in IL-6 among the factors analyzed in this study. At least it may suggest that cows with hypocalcemia have a higher risk of infection in the udder 4 wk after calving. In this study, we used only a quarter of the udder. It is not clear whether the same results would be observed in all quarters. Further studies are needed to clarify the reason for lower IL-6 expression by milk somatic cells from cows with hypocalcemia after calving.

Acknowledgment

The work was supported by a grant from the Meiji Seika Pharma.

References

Experimental evaluation of *Mycoplasma hyopneumoniae* bacterin against a Korean *M. hyopneumoniae* challenge

SooHwan Kim, Taehwan Oh, Siyeon Yang, Hyejean Cho, Chanhee Chae

**Abstract**

The objective of this study was to evaluate the efficacy of a new *Mycoplasma hyopneumoniae* bacterin against a Korean *M. hyopneumoniae* challenge under experimental conditions. Fifteen pigs were allocated randomly into 3 groups (5 pigs per group) that were designated in 1 of 3 ways: vaccinated-challenged, unvaccinated-challenged, or unvaccinated-unchallenged. The pigs in the vaccinated-challenged group were immunized with an *M. hyopneumoniae* whole-cell bacterin at a 1.0 mL dose-level at 21 d old. At 42 d old (0 d post-challenge), the pigs in the vaccinated-challenged and unvaccinated-challenged groups were inoculated intranasally with a strain of Korean *M. hyopneumoniae*. Vaccinated-challenged pigs elicited a strong cell-mediated immunity as measured by *M. hyopneumoniae*-specific interferon-γ secreting cells when compared with unvaccinated-challenged pigs. Vaccination of pigs with this new *M. hyopneumoniae* bacterin reduced nasal shedding and lung lesions. The evaluated vaccine was therefore considered effective in controlling *M. hyopneumoniae* infection.

**Résumé**

L’objectif de cette étude était d’évaluer l’efficacité d’une nouvelle bactérine de *Mycoplasma hyopneumoniae* contre une infection défi

**Mycoplasma hyopneumoniae** infection alone causes relatively mild disease in the absence of environmental stressors, but when complicated by secondary bacterial invaders, may result in obvious clinical disease and severe production losses in intensively reared pigs (1). This respiratory disease is referred to as enzootic pneumonia. *Mycoplasma hyopneumoniae* is probably the most frequent bacterial respiratory infection in pig production and continues to be economically significant worldwide (1).

Vaccination is the most effective strategy for reducing economic losses and the clinical effects of *M. hyopneumoniae* infection on the Asian pork industry. A new single-dose *M. hyopneumoniae* whole-cell bacterin (Hyogen; CEVA Santé Animale, Libourne Cedex, France) was recently introduced into the Asian market to protect pigs against *M. hyopneumoniae* infection. In Europe, the same single-dose *M. hyopneumoniae* whole-cell bacterin provided protection against Belgian *M. hyopneumoniae* field isolates (2). *Mycoplasma hyopneumoniae* field isolates are known to be highly genetic, antigenic, and pathogenically variable between herds and geographical locations (3–5). Moreover, the genetic diversity of *M. hyopneumoniae* field isolates may be one of the factors that affects the efficacy of *M. hyopneumoniae* vaccines (6).

These results strongly suggest that protection of this bacterin against Belgian *M. hyopneumoniae* field isolates does not guarantee the same effective protection against Korean *M. hyopneumoniae* field isolates. The objective of this study was to evaluate the efficacy of the new single-dose *M. hyopneumoniae* whole-cell bacterin (Hyogen; CEVA Santé Animale) based on strain BA 2940–99, oil adjuvanted with paraffin and *Escherichia coli* J5 LPS with thiomersal as excipient, in pigs experimentally infected with *M. hyopneumoniae* for registration as recommended by the Republic of Korea’s Animal, Plant & Fisheries Quarantine & Inspection Agency (QIA), http://qia.go.kr

Unnecessary animal usage was eliminated in accordance with QIA guidelines by selecting and assigning the recommended 5 piglets for each treatment group. A total of 15 colostrum-fed, crossbred, conventional piglets was weaned and purchased at 18 d old from a commercial farm that was free of porcine reproductive and respiratory syndrome virus (PRRSV) and *M. hyopneumoniae* based on serological testing of the breeding herd and long-term clinical and
Table I. Average daily weight gain (ADWG) from 21 to 63 d old and pathology data (mean ± standard deviation) of 5 pigs in each of 3 groups at 21 d post-challenge.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vaccinated-challenged</th>
<th>Unvaccinated-challenged</th>
<th>Unvaccinated-unchallenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADWG</td>
<td>295.71 ± 22.30</td>
<td>291.90 ± 26.76</td>
<td>301.90 ± 16.62</td>
</tr>
<tr>
<td>Macroscopic lung lesion scores</td>
<td>7.3 ± 6.53a</td>
<td>22.7 ± 11.42p</td>
<td>0 ± 0a</td>
</tr>
<tr>
<td>Microscopic lung lesion scores</td>
<td>1.68 ± 0.39a</td>
<td>3.64 ± 0.57p</td>
<td>0 ± 0c</td>
</tr>
</tbody>
</table>

Different superscripts (a, b, and c) indicate significant (P < 0.05) difference among 3 groups.

slaughter history. At 21 d old, sera samples from pigs were found seronegative for porcine circovirus 2 (PCV2), PRRSV, and *M. hyopneumoniae* according to routine serological testing. Sera samples were negative for PCV2 and PRRSV and nasal swabs were negative for *M. hyopneumoniae* when tested by real-time polymerase chain reaction (RT-PCR) (7).

For the study, 15 pigs were allocated into 3 groups (5 pigs per group) using the Excel random number generator function (Microsoft, Redmond, Washington, USA). At −21 d post-challenge (dpc) 21 d old, the pigs in the vaccinated-challenged (Vac/Ch) group were administered a single, 1.0-mL dose of *M. hyopneumoniae* whole-cell bacterin (Hyogen, Lot No. 1405582B; CEVA Santé Animale) intramuscularly based on the manufacturer’s instructions. The pigs in unvaccinated-challenged (UnVac/Ch) and unvaccinated-unchallenged (UnVac/UnCh) groups were administered an equal volume of phosphate-buffered saline (PBS, 0.01 M, pH 7.4, 1.0 mL) at 21 d old. At 0 dpc (42 d old), the pigs in the Vac/Ch and UnVac/Ch groups were inoculated with *M. hyopneumoniae* (strain SNU98703). Infection of pigs with *M. hyopneumoniae* strain SNU98703 caused severe mycoplasmal pneumonia (8).

Pigs in the Vac/Ch and UnVac/Ch groups were anesthetized with a mixture of 2.2 mg/kg body weight (BW) xylazine hydrochloride (Rumpon; Bayer, Leverkusen, Germany), 2.2 mg/kg BW tiletamine hydrochloride, and 2.2 mg/kg BW zolazepam hydrochloride (Zoletil 50; Virbac) by intramuscular injection. Post-anesthetization, pigs were inoculated intratracheally with 7 mL of *M. hyopneumoniae* whole-cell bacterin (Hyogen, Lot No. 1405582B; CEVA Santé Animale) intramuscularly based on the manufacturer’s instructions. The pigs in unvaccinated-challenged (UnVac/Ch) and unvaccinated-unchallenged (UnVac/UnCh) groups were administered an equal volume of phosphate-buffered saline (PBS, 0.01 M, pH 7.4, 1.0 mL) at 21 d old. At 0 dpc (42 d old), the pigs in the Vac/Ch and UnVac/Ch groups were inoculated with *M. hyopneumoniae* (strain SNU98703). Infection of pigs with *M. hyopneumoniae* strain SNU98703 caused severe mycoplasmal pneumonia (8).

Blood and nasal swabs were collected at −21, 0, 7, 14, and 21 dpc. All 15 pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 21 dpc as described in a previous study (9). Tissues were collected from each pig at necropsy. Post-collection, the tissues were fixed for 24 h in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee and Animal Experiment Ethics Committee.

After *M. hyopneumoniae* inoculation, the pigs were monitored daily for physical condition and scored weekly for severity of clinical respiratory disease using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (10). The live weight of each pig was measured at 2 time points throughout the study as follows: −21 (21 d old) and 21 dpc (63 d old). On conclusion of the study, the average daily weight gain ([ADWG] grams/pig per day) was calculated over production stage from 21 to 63 d old. Data for dead or removed pigs were included in the calculation.

Genomic DNA copies of *M. hyopneumoniae* were quantified by real-time quantitative PCR after DNA was extracted from nasal swabs using a commercial kit (QIAamp DNA Mini Kit; QIAGEN, Valencia, California, USA) as described in a previous study (7). Serum samples were tested for antibodies against *M. hyopneumoniae* (M. hyo; Ab test; IDEXX Laboratories, Westbrook, Maine, USA). Serum samples were considered positive for *M. hyopneumoniae* antibodies if the sample-topositive (S/P) ratio was 0.4.

An enzyme-linked immunosorbent (ELISPot) assay was conducted to measure the numbers of *M. hyopneumoniae*-specific interferon-γ secreting cells (IFN-γ-SCs). *Mycoplasma hyopneumoniae* (strain SNU98703) antigens were prepared as described in a previous study (11,12). The numbers of *M. hyopneumoniae*-specific IFN-γ-SCs stimulated by the aforementioned challenge *M. hyopneumoniae* antigen were determined in peripheral blood mononuclear cells (PBMCs) (11,12). The IFN-γ positive spots on the membranes were imaged, analyzed, and counted using an automated ELISPOT Reader (AID ELISPOT Reader; AID GmbH, Strassberg, Germany). The results were expressed as the numbers of IFN-γ-SCs per million PBMCs. The ELISPOT assay was done in duplicate.

Morphometric analysis of the macroscopic pulmonary lesion was scored on a total scale of 100 points as follows: 10 points each to the right cranial lobe, right middle lobe, left cranial lobe, and left middle lobe; 27.5 points each to the right caudal lobe and left caudal lobe; and 5 points to the accessory lobe (10). Microscopic mycoplasmal pulmonary lesions were scored (0 to 6) based on the severity of peribronchiolar and perivascular lymphoid tissue hyperplasia (13). All lung section scoring was evaluated blindly by 2 pathologists.

Prior to statistical analysis, RT-PCR data were transformed to log₁₀ values. Data were tested for normal distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to examine whether there were statistically significant differences at each time point within the 3 groups. A 1-way ANOVA test result with such a statistical significance was further evaluated by conducting a post-hoc test for a pairwise comparison with Tukey’s adjustment. If the normality assumption was not met, the Kruskal-Wallis test was conducted. A result from the Kruskal-Wallis test that showed statistical significance was further evaluated with the Mann-Whitney test to include Tukey’s adjustment to compare the differences among the groups. Results were reported in *P*-value in which a value of *P* < 0.05 was considered to be significant.
The mean scores for respiratory disease were significantly lower ($P < 0.05$) in pigs from the Vac/Ch group when compared to the UnVac/Ch group at 14 and 21 dpc. The pigs from the UnVac/UnCh group remained normal throughout the experiment. There was no significant difference in ADWG among the 3 groups from 21 to 63 d old (Table I).

Pigs in the Vac/Ch group had significantly less ($P < 0.05$) $M. \text{hyopneumoniae}$ genomic copies in their nasal swabs compared to the UnVac/Ch group at 14 and 21 dpc (Figure 1). No $M. \text{hyopneumoniae}$ was detected in the pigs from the UnVac/UnCh group.

Pigs in the Vac/Ch group had a significantly higher ($P < 0.05$) $M. \text{hyopneumoniae}$ enzyme-linked immunosorbent assay (ELISA) S/P ratio in their serum samples when compared with the UnVac/Ch group from 0 to 7 dpc (Figure 2), as well as a significantly higher number of $M. \text{hyopneumoniae}$-specific interferon-$\gamma$-secreting cells (IFN-$\gamma$-SCs) in their PBMCs (Figure 3) when compared with the UnVac/Ch group from 0 to 21 dpc. No $M. \text{hyopneumoniae}$-specific antibodies and IFN-$\gamma$-SCs were detected in pigs from the UnVac/UnCh group.

Pigs in the Vac/Ch group had significantly lower ($P < 0.05$) macroscopic and microscopic lung lesion scores when compared with the

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The results of the present study demonstrate that vaccinated-challenged pigs develop fewer lung lesions and nasal route excretion than unvaccinated-challenged pigs. This variance between the 2 groups is probably due to differences in protective immunity. Protective immunity against *M. hyopneumoniae* is not fully understood. The fact that the pathogen is non-invasive, but can still induce pneumonia, implies that cellular immune response plays a significant role (14,15). Vaccinated-challenged pigs elicited a strong cell-mediated immunity as measured by *M. hyopneumoniae*-specific IFN-γ-SCs when compared with unvaccinated-challenged pigs. Induction of cell-mediated immunity by *M. hyopneumoniae* vaccine plays a significant role in protecting pigs against *M. hyopneumoniae* infection, as implied by previous studies (12).

There are 2 ways to assess the efficacy of vaccines: field clinical and experimental challenge trials. Field clinical trials are suitable for evaluating pig productivity. Vaccination against *M. hyopneumoniae* improved pig productivity and was reported as increased growth performance and decreased mortality under field conditions (16–20). Despite vaccination efforts, *M. hyopneumoniae* continues to circulate within pig herds, leading to the possibility of exposure and re-exposure to the virus by horizontal transmission under field conditions. Meanwhile, experimental challenge trials are suitable for microbiological, immunological, and pathological evaluation.

Growth performance was also evaluated in the present experimental challenge study. There was no significant difference in ADWG between vaccinated-challenged and unvaccinated-challenged groups because of the small number of pigs in each group and the short duration observed after challenge with *M. hyopneumoniae*. These results agree with a previous study in which the same vaccine showed no significant difference in growth performance under experimental conditions (3). Nevertheless, vaccination of pigs with this newly evaluated *M. hyopneumoniae* bacterin benefits the pig by eliciting cell-mediated immunity and reducing nasal shedding and lung lesions. The newly evaluated vaccine may therefore be an effective tool in controlling *M. hyopneumoniae* infection.

**Acknowledgments**

The authors’ research was supported by contract research funds (Grant no. 550-20180057) from the Research Institute for Veterinary Science (RIVS) of the College of Veterinary Medicine at Seoul National University and by the BK 21 FOUR Future Veterinary Medicine Leading Education and Research Center.

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Contact/Communiquez avec :
Kelly Gray-Sabourin
Editorial Coordinator/Coordonnatrice de la rédaction
Canadian Journal of Veterinary Research
Revue canadienne de recherche vétérinaire
339 rue Booth Street
Ottawa, Ontario K1R 7K1
Canada
Tel./Tél. : (613) 236-1162, x117
Fax/Télécopieur : (613) 236-9681
E-mail/Courriel : kgray@cvma-acmv.org

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