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Canadian Journal of Veterinary Research

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**Introduction**

Osteoarthritis (OA) is a degenerative joint disease affecting 60% of horses (1). Progressive destruction and loss of articular cartilage due to limited ability of this structure to repair itself are the central features in OA (1,2). Osteoarthritis is a multifactorial disease that can start with an injury in any component of a joint (1,3). Fibroblast-like synoviocytes (FLSs) or type B synoviocytes can produce different proteolytic enzymes, cytokines, chemokines, and growth factors that eventually lead to cartilage damage and destruction (1,4-6). Matrix metalloproteinases (MMPs) are important, because of their critical role in degradation of extracellular matrix proteins comprising of joint tissues, including articular cartilage, bone, intra-articular ligaments, and tendons (6-8). Elevation of levels and activities of different MMPs have been reported in serum and synovial fluid of arthritic diseases, as well as acute phase proteins (9-12). Serum amyloid A (SAA) proteins are the major acute-phase proteins that are released during acute phase in response to infection, inflammation, and trauma (11-13). Serum amyloid A is synthesized and secreted primarily in the liver but local expression of SAA has been detected in synoviocytes and chondrocytes from bone and cartilage in arthritis (11-14). Serum amyloid A is linked to the pathogenesis of arthritic disease and progressive joint damage (15-17). Despite advances in diagnostic methods for arthritis, the therapeutic protocols have been

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**Abstract**

Application of synthetic matrix metalloproteinases (MMPs) inhibitors, such as doxycycline is one of the possible therapeutic options for osteoarthritis. However, little is known about the protective mechanism of doxycycline in equine models on MMPs inhibitors as well as on serum amyloid A (SAA) gene expression. This study investigated the effects of doxycycline on mRNA expression of MMP-1, MMP-2, MMP-9, MMP-13, and SAA of equine fibroblast-like synoviocytes (FLSs). The FLSs were established from synovial fluids of clinically normal metacarpophalangeal joints of 6 skeletal mature horses. The cells were treated with either 10 or 100 µg/mL of doxycycline for 48 h. The mRNA expression of MMP-1, MMP-2, MMP-9, MMP-13, and SAA were assessed using real-time polymerase chain reaction (PCR). Treatment with doxycycline resulted in significantly decreased mRNA expression of MMP-1 in FLSs at both concentrations (P = 0.001). No significant differences were detected among groups for MMP-2, MMP-9, and MMP-13 (P > 0.05). Only a tendency towards a decrease in mRNA expression level of SAA in the presence of doxycycline could be detected. Doxycycline inhibits MMP-1 gene expression at the transcript level. These findings indicate that doxycycline can protect the articular environment through inhibition of MMP-1 at transcript level.
limited to manage its symptoms (1,18). Because of the fundamental functions of FLSs and their biologically active products MMPs and SAA in degradation and remodeling of extracellular matrix both in healthy and arthritic joints, they are considered valuable agents for therapeutic targets (4,7–9,17).

Doxycycline is a semi-synthetic tetracycline antibiotic with a broad spectrum of activity (19). Besides anti-microbial function, doxycycline is also anti-inflammatory, antioxidant, antitumor, immunomodulatory, and MMP inhibitor and could be a reasonable candidate for the treatment of different pathologic conditions (20,21). Several direct and indirect mechanisms have been suggested for inhibitory activity of doxycycline on MMPs production and activity (22–24).

Although the inhibitory role of doxycycline on MMPs has been investigated in in vitro and in vivo studies (22), these data are limited in horses and additional studies are required to evaluate whether doxycycline might be effective in the treatment of arthritic joints in horses. To our knowledge, there are limited studies about SAA in equine joints and no study to date has investigated the effect of doxycycline on SAA gene expression in synovial cell culture. Our in vitro design was designed to investigate whether doxycycline has an effect on genes expression of MMP-1, MMP-2, MMP-9, MMP-13, and SAA in equine FLSs.

### Materials and methods

#### Animals

Six skeletally mature horses, 4- to 7-years-old, of different breeds with no history of orthopedic disease according to their owners were selected for this study. These horses were examined and confirmed to be free from lameness by clinical examinations and had clinically normal metacarpophalangeal joints by radiographs.

#### Isolation and culture of FLSs from synovial fluid

Approximately 4 to 5 mL of synovial fluid samples from the metacarpophalangeal joints were taken using syringes containing sodium heparin (Heparodic, Caspianamin, Gilan, Iran) with 19-gauge needles under aseptic conditions. All collected synovial fluids were reported normal in quantity, viscosity, and color by laboratory report. To isolate FLSs from the fresh synovial fluids, samples were diluted with equal volumes of phosphate buffered saline (PBS; Gibco BRL, Eggenstein, Germany) and centrifuged at 600 × g for 30 min. The cell pellets from each synovial fluid sample were then resuspended in Dulbecco’s Modified Eagle’s Medium low glucose (DMEM; Gibco BRL), supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% penicillin streptomycin solution (Gibco BRL), and were seeded into 12-mm culture plates. Subsequent passages of FLSs were done until 5th passage. The growth of FLSs was monitored using light microscope until observance of 70% cellular confluence. All plates had similar cell densities at this point.

#### RNA extraction and cDNA synthesis

RNA extraction from FLSs was done using a reagent (AccuZol; Bioneer, Seoul, South Korea) according to the manufacturer’s instructions. The extracted RNA was dissolved in diethylpyrocarbonate-treated (DEPC; SinaClon BioScience, Karaj, Iran) water. RNase-free DNase (SinaClon BioScience) was added to total RNA to clean possible contaminating genomic DNA. The purified total RNA was analyzed by agarose gel electrophoresis and spectrophotometry. Only RNA samples with the absorbance ratio (A260/280) between 1.8 and 2, and 2 distinct bands representing the 28S and 18S rRNA were considered for synthesis of cDNA. Total extracted RNA (1 μg) was reverse transcribed to cDNA a short time after extraction in a 20 μL reaction volume using a ready-to-use reverse transcription kit AccuPower CycleScript RT PreMix (AccuPower Cycle Script RT PreMix; Bioneer, Daejeon, South Korea) following the manufacturer’s instructions. Thermal program for cDNA synthesis included the following 3 steps in 12 cycles: 25°C for 30 s, 45°C for 4 min, and 55°C for 30 s. The cDNA mix was heated to 95°C for 5 min to inactivate the reaction and denature the RNA. After this step, PCR was done using primers for equine GAPDH to control the presence of cDNA in the prepared samples.

#### Quantitative real-time PCR

The FLSs mRNA was evaluated for expression of MMP-1, MMP-2, MMP-9, MMP-13, and SAA. The level of gene transcripts was determined by real-time PCR using SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan). This method required a suitable internal standard to control for variability between samples and to normalize the input load of cDNA. For this purpose, gene transcript level and stability of 6 candidate reference genes (ACTB, GAPDH, HPRT1, IPO8, PPIA, MRPL19) were determined in equine FLSs using 3 software packages geNorm (Center for Medical Genetics, Ghent University,
Table 1. Primers used for quantitative real-time polymerase chain reaction (PCR) analysis to detect expression of mRNA cultured equine fibroblast-like synoviocytes (FLSs).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
<th>PCR product</th>
<th>Accession no</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>GGTGGGAGAACACACACCTGT TCCGCTTAGCCCTAGAGAC</td>
<td>151</td>
<td>NM_001081847.2</td>
</tr>
<tr>
<td>MMP-2</td>
<td>GCCAATCAATCGAGCACCCGG GGGAATGTGTCGCTTACG</td>
<td>162</td>
<td>XM_014738411.1</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CGGCCCTATGAGCAGGTCG CTGGTCGTAGTGGCCTTAG</td>
<td>191</td>
<td>NM_00111302.1</td>
</tr>
<tr>
<td>MMP-13</td>
<td>GCTGGACTGTGTTGGTTCC CTGTCACACCAGGATCG</td>
<td>167</td>
<td>NM_001081804.1</td>
</tr>
<tr>
<td>SAA</td>
<td>CGGGTGCCAGGGCTTTGTT GCAGGGGGGTTGGAATTTG</td>
<td>127</td>
<td>AF240364.1</td>
</tr>
<tr>
<td>GAPDHb</td>
<td>GCGAGATCCGCCAATCT GTGCCACCTCCATTGAGG</td>
<td>111</td>
<td>NM_1163856.1</td>
</tr>
<tr>
<td>HPRT1b</td>
<td>GAGCCGCAGTTGCTGATT CATCTGAGCAAGGCTTCT</td>
<td>146</td>
<td>XM_014729196.1</td>
</tr>
<tr>
<td>PPIACc</td>
<td>GTCCATGGGCAAGTCTGCC CCATCCCGAGCGCAACAGG</td>
<td>156</td>
<td>XM_001496943.4</td>
</tr>
</tbody>
</table>

a Glyceraldehyde-3-phosphate dehydrogenase.
b Hypoxanthine phosphoribosyl transferase 1.
c Peptidylprolyl isomerase.

Results

Quantitative real-time PCR analysis revealed that MMP-1, MMP-2, MMP-9, MMP-13, and SAA mRNA were expressed by clinically normal equine FLSs. The relative transcript of MMP-1 was downregulated after doxycycline treatment for 48 h, \( P = 0.001 \), \( P < 0.05 \). Doxycycline at both concentrations decreased mRNA expression of MMP-1. Pairwise comparison revealed that there was a significant difference for MMP-1 expression between each treatment group (10 and 100 \( \mu \text{g/mL} \) doxycycline) compared with control group. The relative amount of MMP-1 mRNA expression significantly decreased (72.6\%, \( P = 0.004 \), \( P < 0.017 \) and (82.08\%, \( P = 0.002 \), \( P < 0.017 \)) in 10 and 100 \( \mu \text{g/mL} \) doxycycline, respectively, compared with the control group (Figure 1A). The ratio of MMP-1 mRNA expression in 10 and 100 \( \mu \text{g/mL} \) doxycycline treated groups compared to the control group were 0.27 and 0.20, respectively. No significant difference was found between the 2 concentrations of doxycycline for MMP-1 expression.

No significant differences for MMP-2, MMP-9, and MMP-13 mRNA expression were noted at the 2 concentrations of doxycycline compared to the control group (\( P > 0.05 \); Figure 1B-D). The expression of SAA mRNA was detected in FLSs in all 3 groups. There was a tendency to measure lower SAA levels by increasing doxycycline concentration but, there was no statistical significance (\( P > 0.05 \); Figure 1E).

Discussion

Different MMPs including MMP-1 (collagenase 1), MMP-13 (collagenase 3) MMP-2 (gelatinase A), and MMP-9 (gelatinase B), are
produced by synoviocytes and have been suggested as key players in destroying the structural collagens as well as non-collagen matrix molecules in arthritic joints (6,8,28,29). Doxycycline is one of the agents, referred to as disease-modifying OA drugs (DMOADs) with several other effects, which are independent from its antimicrobial activities and is now the subject of preclinical and clinical trials (1,23,30). The inhibition of MMPs expression by doxycycline occurs both at mRNA and at protein levels (23,24). Oral administration of doxycycline for treatment of osteoarthritis is not routinely used in horses due to some side-effects such as photosensitization, intestinal flora disruption, and concerns of antibiotic resistance (31). The intra-articular method for administration of therapeutic drugs for osteoarthritis treatment has several advantages over systemic delivery (32). It seems intra-articular administration of doxycycline may be better to use in OA treatment, because it delivers doxycycline to the joint cavity directly and minimizes the related adverse systemic effects. Before it is possible to recommend intra-articular of doxycycline for clinical use, in vitro studies are necessary to understand its exact mechanism and efficiency on all components of the joint.

As the FLSs are active component in the maintenance of joints as well as in osteoarthritis pathology, this study evaluated the effects of doxycycline on MMPs expression in FLSs.

The inhibition of MMP-1 as a multifunctional MMP may contribute to slow down the progress of osteoarthritis. The data presented here show that at both concentrations of doxycycline (10 and 100 μg/mL), expression of MMP-1 was significantly reduced, whereas there was no significant reduction in the expression of MMP-2, MMP-9, and MMP-13. These results suggest that

---

**Figure 1.** The effects of doxycycline on relative mRNA expression of matrix metalloproteinase (MMP-1) (A), MMP-13 (B), MMP-2 (C), MMP-9 (D), and serum amyloid A (SAA) (E). Cultures were treated without and with doxycycline (10 or 100 μg/mL) for 48 h. Statistically significant difference (P < 0.05, P = 0.001) was only in MMP-1 (A). Data for all groups are presented in mean ± SD. a,b Represents changes that are significantly differences between the 2 groups.
doxycycline has an inhibitory effect on MMP-1, but not on MMP-2, MMP-9, and MMP-13 at transcript level. Therefore, doxycycline does not have the same inhibitory mechanism on expression of different types of MMPs in FLSs. This effect of doxycycline on MMP-1 expression in the present study, appears to be in agreement with Axisa et al (33). They found that doxycycline significantly reduced MMP-1 gene transcript, but not MMP-2 and MMP-9 (33). It has also been shown that incubation of human OA and normal chondrocytes with doxycycline for 24 h at concentrations of 1, 10, and 50 µg/mL was able to down-regulate MMP-1 and MMP-13 mRNAs and proteins (34). In contrast, a study on bovine chondrocytes, found no inhibition of mRNA MMP-1 expression after use of 10 µM (\(~4.44\) µg/mL) doxycycline for 48 h by RT-PCR-ELISA method (35). Some differences between this study and our study include the use of bovine chondrocytes versus equine FLSs, the differences between used doxycycline concentrations, and RT-PCR-ELISA versus real-time PCR for detection of MMP-1 mRNA expression, which might explain these contrasting results.

The results of our study show that doxycycline had no significant effect on MMP-2 and MMP-9 mRNAs expression. Matrix metalloproteinase-2 and MMP-9 are important in the evolution of joint erosions and degradation in arthritic joints (8,36,37). No experiments have evaluated the effectiveness of doxycycline on MMP-2 and MMP-9 expression in FLSs or chondrocytes, based on the authors’ knowledge. Although, some studies have investigated the effects of doxycycline on MMP-2 and MMP-9 in other kinds of cells including human squamous-cell carcinoma, aortic smooth muscle cells, glioblastoma, and leukemic cells (38–41). Reduced expression of MMP-9 at the transcriptional and MMP-2 at the post-transcriptional levels have been reported after treatment with 10 µg/mL of doxycycline in human lingual squamous-cell carcinoma cell lines (38). In another study, doxycycline had an effect on MMP-2 post-transcriptionally by reducing MMP-2 mRNA half-life and stability in aortic smooth muscle cells (39). Although these studies have revealed inhibitory effects of doxycycline on MMP-2 and MMP-9 expression, in our study there was no direct effect of doxycycline on MMP-2 and MMP-9 mRNAs expression. However, further studies are needed to determine whether doxycycline can affect MMP-2 and MMP-9 expressions at the post-transcriptional level such as mRNA stability or translation in FLSs. In contrast to the studies have suggested that doxycycline decreases MMP-2 and MMP-9 expression (38–41), in other some studies, over transcription of MMP-2 and MMP-9 with doxycycline have been reported in some cell lines of glioblastoma and leukemic cells, interestingly. This may be related to cellular genetic differences or due to cellular stress response which activated MMP-2 and MMP-9 genes transcription (40,41).

Matrix metalloproteinase-13 is considered as one of the key MMPs with a major role in cleaving type II collagen in OA (8,36). Synoviocytes and chondrocytes from clinically normal and arthritic joints can express MMP-13 (24). It has been suggested that MMP-13 is more sensitive to doxycycline than other MMPs (42). Maher and colleagues (42) reported that MMP-13 gene expression was significantly decreased in pre-treated synoviocytes with interleukin-1 (IL-1) cultured in medium containing doxycycline (0.025 to 0.105 µg/mL) after 48 h. Fortier et al (24) used different concentrations of doxycycline (0.043, 0.43, 4.3 µM: ~0.022, ~0.22, ~2.2 µg/mL) in equine synoviocytes and chondrocytes for 96 h. They reported that doxycycline at 4.3 µM (~2.2 µg/mL) inhibited MMP-13 mRNA expression only on synoviocytes (24). In contrast to those studies mentioned above, an in vitro arthritis study documented that MMP-13 was relatively more resistant than MMP-1 to doxycycline, and doxycycline inhibited MMP-13 starting at approximately 200 µM (~104 µg/mL) (43). The inhibition of MMP-13 mRNA and protein expression by high doses of doxycycline was reported in chondrocytes culture (44). In comparison with the above studies, we used 10 and 100 µg/mL dosages of doxycycline for 48 h and could not establish any significant difference in MMP-13 mRNA expression. In addition to the concentration of doxycycline, the length of time of drug exposure may have affected the MMP-13 expression. The significant reduction of MMP-13 gene expression by treatment of doxycycline has been reported in a time-dependent manner in human bone marrow-derived mesenchymal stem cells chondrogenic cultures at 21 d (45).

Collectively, evaluation of the available studies and our study have shown that doxycycline affects expression of different MMPs in a wide concentration range at mRNA or protein levels. This may be depended on the specific MMP tested (34). Hence, detection of exact dosage of doxycycline for MMPs inhibition is difficult. These differences observed among various studies may be related to the variations in experimental conditions. Some studies were done in vitro and others in vivo where diverse pathways can mediate the response of MMPs genes to doxycycline (46). Different types of cells from different origins have been used as models for these doxycycline studies. Thus, the types of cell used can influence the expression of MMPs treated with doxycycline. The differences in the permeability and retentivity for doxycycline, discrepancy in genetics, and metabolic potentials of cells, as well as diversity of expression pattern of various MMPs in cells may be the possible reasons behind these challenges.

In conclusion, although it was confirmed that doxycycline suppresses different matrix metalloproteinases expression, the data presented here indicate that doxycycline was only able to inhibit MMP-1 mRNA expression in equine FLSs.

In this study, the expression of SAA in equine FLSs and the impact of doxycycline on its mRNA were investigated. In joints, SAA produced by synoviocytes and chondrocytes are proposed as the sources of synovial fluid SAA (12). In vitro and ex vivo culture models have demonstrated that SAA has a potent ability to induce MMP-1, MMP-3, MMP-2, MMP-9, and MMP-13 production as well as IL-1β and tumor necrosis factor-α (TNF-α) (11,17). Little information is available about SAA expression in equine joints. In the study presented here, SAA mRNA was detected in clinically normal equine FLSs. Our results appeared to be contradictory to some findings in the literature that suggested SAA is synthesized in inflamed joint cells (11,12). However, there is some evidence showing that SAA is produced in clinically normal FLSs and chondrocytes (5,14). Low to moderate expression of SAA mRNA was detected in histologically normal synovial membrane of horses and synoviocytes obtained from clinically normal equine metacarpophalangeal joints (47,48). In another study, it was revealed that expression levels of SAA were significantly different in normal chondrocytes cultured from different joints of horses (49). No study to date has described alterations in
SAA mRNA expression in cultured FLSs treated with doxycycline. There was a tendency to diminish the level of SAA mRNA following doxycycline treatment in equine FLSs in our study, but this change was not significant.

In summary, the present study concludes that doxycycline may have a protective potential in equine joints. This effect mainly is associated with inhibition of MMP-1 expression at transcript level. However, this short-term study has some limitations, further investigations are recommended to evaluate the efficacy of doxycycline in equine osteoarthritis.

Acknowledgment

Financial support for this study was provided by the Ferdowsi University of Mashhad.

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Using a computer simulation model to examine the impact of biosecurity measures during a facility-level outbreak of equine influenza

Kelsey L. Spence, Terri L. O’Sullivan, Zvonimir Poljak, Amy L. Greer

Abstract

On-farm biosecurity measures are an important part of a control plan to minimize the introduction and spread of infectious diseases, such as equine influenza, in an equine facility. It can be challenging, however, to evaluate the efficacy of biosecurity measures under field conditions. We used an agent-based computer simulation model to describe the impact of: i) preventive vaccination; ii) reduced horse-to-horse contact; and iii) a combination of vaccination and reduced contact during an outbreak of equine influenza in a simulated horse facility. The model demonstrated that the most effective intervention was a combination of a high proportion of recently vaccinated horses and a substantial reduction in horse-to-horse contact once equine influenza had been identified in the facility. This study highlights the importance of compliance when implementing biosecurity measures, such as facility-level infection control practices, on horse farms.

Résumé

Les mesures de biosécurité à la ferme sont une composante importante d’un plan de maîtrise afin de minimiser l’introduction et la dissémination de maladies infectieuses, telle que l’influenza, dans une installation équine. L’évaluation de l’efficacité de mesures de biosécurité dans des conditions de champ peut toutefois représenter un défi. En absence d’une épidémie active, nous avons utilisé une simulation informatisée d’une infection afin de décrire l’impact de : i) une vaccination préventive; ii) une réduction des contacts cheval-à-cheval; et iii) une combinaison de vaccination et de contacts réduits sur le taux d’attaque projeté d’une épidémie simulée d’influenza équin dans un établissement. Les résultats ont démontré que l’intervention la plus efficace était une combinaison d’une proportion élevée de chevaux récemment vaccinés et d’une réduction substantielle des contacts cheval-à-cheval une fois que l’influenza fut identifiée dans l’établissement. Cette étude fait ressortir l’importance de la compliance lors de la mise en place de mesures de biosécurité sur des fermes équines, telles que des pratiques de maitrise des infections au niveau de l’établissement.

Introduction

Equine influenza (EI) is a respiratory disease caused by an influenza A virus [equine influenza virus (EIV)] and is common in equine populations worldwide (1,2). Clinical signs of EI include fever, depression, nasal discharge, and coughing (1), all of which impair athletic performance (3). Equine influenza virus is directly transmitted through close contact with aerosolized respiratory droplets and indirectly transmitted through contact with contaminated equipment, food/water, vehicles, and the hands/clothing of humans (1,4). The highly contagious nature of EIV and its ability to spread rapidly in susceptible populations contribute to its economic importance at the regional, national, and international level (2).

The potential impact of EI within an equine facility can be minimized by implementing infection prevention and control measures. Prevention measures, such as quarantine after travel, stabilizing in well ventilated facilities, and vaccination, are used to prevent the establishment of EI (5–7). While vaccination against EI is mandatory in some populations (2), it is not mandatory in Canada (8). Vaccination is recommended, however, for horses at higher risk of exposure to infection, such as young and older horses, breeding horses, and those that travel frequently (9). While vaccination may offer short-term immunity, variations in immunological response, vaccination regimens, and duration of immunity affect population-level disease dynamics (5,10). Other biosecurity measures should be used in addition to vaccination to minimize the risk of introducing EI and its subsequent spread. These measures include quarantine after travel, reducing contact between horses, and infection control strategies implemented by equine facility staff, such as hand-washing (5–7).

The successful implementation of biosecurity and infection control measures relies on the compliance of horse owners and equine facility staff. In the United States, a survey of horse owners revealed that 80% of respondents either did not use biosecurity measures or were not familiar with the biosecurity measures used at their horse’s facility (11). Similarly, a survey of horse facilities in New Zealand demonstrated that the general usage of biosecurity measures at the facilities would be insufficient to prevent the introduction of disease (12). The lack of awareness about the benefits of biosecurity and infection control measures suggests that alternate strategies are required to demonstrate their importance. Furthermore, quantifying the effects of biosecurity and infection control measures can strengthen the existing recommendations for the Canadian equine industry (13).

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Received March 28, 2017. Accepted July 29, 2017.
A computer simulation model is a tool that can synthesize knowledge from the peer-reviewed literature to capture the expected disease dynamics within an average population without the use of clinical trials (14). Models can provide critical insight into population-level effectiveness for interventions that may not be practical or cost-effective to implement without stronger evidence (14). Key features of EI outbreaks have previously been described using mathematical models. These features include estimating the basic reproductive number ($R_0$) of equine influenza (15,16), evaluating disease control strategies during an outbreak (17–19), and optimizing recommended vaccination strategies (20,21). However, these previous studies have focused on disease control in exotic outbreak situations or in specific populations of horses, for example, Thoroughbred racehorses in training.

The objective of this study was to use a computer simulation model to describe the impact of biosecurity and infection control measures during an EI outbreak within a simulated equine facility. This study focused on the effect of: i) increased initial facility-level vaccine coverage before EI is introduced; ii) reduced horse-to-horse contact after EI has been identified; and iii) a combination of increased vaccine coverage and reduced horse-to-horse contact. We hypothesized that implementing increased vaccine coverage before the outbreak and reduced horse-to-horse contact during the outbreak would greatly minimize the extent of the outbreak compared to either intervention alone.

### Materials and methods

#### Model description

A stochastic agent-based model was constructed using Anylogic 7.1.2 (XJ Technologies, St. Petersburg, Russia) and was parameterized using data from the peer-reviewed literature (Table I). Agent-based models describe individual ‘agents’ in a population to account for biological variability and potential differences in behavior. We chose an agent-based model because we could explicitly describe each horse’s vaccination status and the associated time since vaccination to account for individual-level heterogeneity in waning immunity.

#### Population structure

We generated a synthetic population of 100 horses housed within a single equine facility. Since we were interested in examining short-term disease dynamics following a disease introduction event, we assumed the facility was a closed population with no births or deaths and no incoming or outgoing movements, as might be expected once an EI outbreak was identified. Individual horses were assigned to distinct age categories, with 35% of the population being yearlings (<2 y old) and 65% being non-yearlings (≥2 y old), to account for differences in vaccine coverage between young and adult horses. The proportion of horses in each age category was chosen to capture the observation that an average equine facility has a higher proportion of non-yearlings than yearlings (T. O’Sullivan, personal communication, 2017).

#### Calibration

The model was calibrated to estimate the contact rate from an EI outbreak reported in the literature. The contact rate was defined as the number of horses that a single horse adequately contacts per day, i.e., a close proximity contact that would be suitable for EIV transmission (22). We assumed that contact was homogenous and all horses had an equal probability of randomly contacting one another. Furthermore, only the effects of EIV transmission via direct horse-to-horse contact were considered. Data from Morton et al (23) were used for model calibration. Morton et al (23) describe a closed population of susceptible horses during the 2007 EI outbreak in Queensland, Australia. Our model was calibrated by varying the contact rate across a wide range of realistic values and determining the parameter value that provided the best fit to the data using least squares estimation.

In order to accurately compare the 2 populations, the outbreak curve reported by Morton et al (23) was converted to the number of new cases per day, divided by the total population size, and multiplied by 100. Vaccination (and waning immunity) was not included as a compartment in the calibration model and all horses were treated as susceptible during the calibration process. The calibration experiment was run for 1000 iterations to account for stochasticity due to the disease initialization process.

#### Vaccination and waning immunity

We assumed that 50% of yearlings and 70% of non-yearlings were vaccinated at the beginning of the simulation, corresponding to an initial facility-level vaccine coverage of 63% (Figure 1). Since we were interested in observing the effect of increasing the initial vaccine coverage, we assumed that these proportions would represent a scenario with a moderate level of initial vaccine coverage, with yearlings having lower vaccine coverage than non-yearlings. Age was only used to determine the initial proportion of vaccinated horses within each category, aging between categories was not considered because horses were not revaccinated during the outbreak. We assumed that vaccination in the facility was synchronized for all horses and occurred before the start of the model. Furthermore, we assumed that vaccinated horses had virological protection, either intervention alone.

#### Table I. Disease parameters used in the agent-based model of an equine influenza outbreak in a simulated equine facility.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact rate</td>
<td>4.86 contacts/day</td>
<td>Model calibration</td>
</tr>
<tr>
<td>Probability of transmission given contact</td>
<td>1.00</td>
<td>(20)</td>
</tr>
<tr>
<td>Latent period</td>
<td>1.25 days</td>
<td>(15)</td>
</tr>
<tr>
<td>Infectious period</td>
<td>5.5 days</td>
<td>(15)</td>
</tr>
<tr>
<td>Period of virological protection</td>
<td>50 days</td>
<td>(26)</td>
</tr>
<tr>
<td>Waning immunity following vaccination</td>
<td>Triangular distribution (Min. 51 days; max. 180 days; mode 116 days)</td>
<td>(26)</td>
</tr>
</tbody>
</table>
which was defined as protection against transmitting and becoming infected with EIV. While challenge studies have demonstrated that previously vaccinated horses have a reduced probability of becoming infectious (20) and a reduced period of infectiousness (20,24,25), we assumed that the contribution of partially vaccinated horses to the force of infection would be negligible in our small population of horses. Furthermore, we did not consider the effect of a mismatched vaccine due to differences between the vaccine strain and the circulating EIV strain. For these reasons, we assumed that vaccinated horses could not transmit or become infected with EIV.

Stochasticity in the model was introduced using a triangular distribution to represent waning immunity after vaccination (Table I). This distribution described the time at which horses in the vaccinated compartment returned to the susceptible compartment as their vaccine-induced immunity waned over time. Assumptions about the waning immunity distribution in the model were informed using results described by Newton et al (26). The immune response to EIV infection leads to antibody production that is adequate for virological protection for a short period of time post-vaccination, after which individual-level variability in the duration of virological protection is expected (26). In the model, all vaccinated horses were considered to have virological protection for at least 51 d and potentially for as long as 180 d. After 51 d, horses began to lose immunity at a rate defined by the waning immunity distribution and re-entered the susceptible compartment. This meant that vaccinated horses could re-enter the susceptible compartment at any point between 51 and 180 d, but most did so at the midpoint between these time points (116 d). Although waning immunity also occurs following natural infection with EIV, it was not incorporated into the model structure because the period of waning immunity following natural infection is longer than the model duration (27).

**EI introduction**

Three independent model scenarios were chosen to represent different levels of risk in a population as vaccine-induced immunity wanes over time. In the ‘low-risk scenario,’ 1 infectious horse was introduced 4 mo after vaccination, at a time when most previously vaccinated horses in the population would be susceptible to infection due to waning immunity. One time-step represented 1 d and the model was run for 180 d.

The EI disease process followed a design based on 5 compartments: ‘susceptible,’ ‘exposed,’ ‘infected,’ ‘recovered,’ and ‘vaccinated,’ which are shown in Figure 1. Each horse in the population could be in only 1 compartment at any time during the simulation and horses transitioned between the compartments according to the parameters listed in Table I. Unvaccinated horses began the simulation in the susceptible compartment. Equine influenza virus (EIV) was transmitted between an infected horse and a susceptible horse through direct contact, at a rate determined through the calibration experiment, and was a function of both the contact rate and the probability of transmission given an adequate contact. Newly infected horses remained in the ‘exposed’ compartment for the duration of the latent period (Table I) and could not transmit EIV while in this compartment. We assumed that horses in the ‘infected’ compartment were equally infectious and remained so for the duration of the infectious period (Table I).

**Model outcomes**

Each scenario was run for 1000 iterations to calculate a range of possible attack rates in the absence of any additional interventions. This was referred to as the ‘base case scenario.’ The clinical attack rate was defined as the total number of infected horses at the end of the simulation run, divided by the size of the population (100 horses). The secondary outcome of interest was the outbreak duration, which was defined as the time when the outbreak ended. Model outcomes were summarized using Stata Statistical Software, Release 14 (StataCorp, College Station, Texas, USA) and the ‘ggplot2’ package in R (R Core Team, Vienna, Austria).

**Interventions**

Three intervention strategies were tested in each scenario: i) increases in the initial facility-level vaccine coverage to 73%, 83%,
and 93% before the outbreak; ii) decreases in the contact rate by 25%, 50%, and 75% during the outbreak, referred to as a reduction in horse-to-horse contact; and iii) a combination of increased vaccine coverage and reduced horse-to-horse contact, referred to as bundled interventions, which are further described in Table II. An increase in the initial facility-level vaccine coverage was directly proportional to the proportion of horses that would have vaccine-induced immunity at the beginning of the model. For example, at 93% vaccine coverage, 93% of the horses in the facility would have vaccine-induced immunity at the start of the simulation. We assumed that it would take 1 d to implement a reduction in horse-to-horse contact after a single case of EI was identified. Horse-to-horse contact was therefore reduced the day after the infectious horse was introduced into the facility. Each intervention scenario was run for 1000 iterations due to model stochasticity.

Sensitivity analysis

Univariate sensitivity analyses were conducted to determine the impact of assumptions regarding the infectious period and the waning immunity distribution. Each parameter was varied independently for 1000 iterations per value (Table III). The infectious period was varied between 3.0 d and 7.0 d, consistent with the previously reported EI shedding period (27). The waning immunity distribution was varied to determine the impact of immunity waning 30 d earlier than expected and 30 d later than expected.

Results

Model calibration and base case outcomes

Calibration to outbreak data (23) resulted in a best-fit contact rate parameter of 4.86 contacts per day, which was used for all examined model scenarios. In the low-risk base case scenario, the average clinical attack rate was 38% (SD = 5%, range: 21% to 58%) and the average outbreak duration was 15 d (SD = 6 d, range: 6 to 23 d). In the medium-risk base case scenario, the average clinical attack rate was 38% (SD = 5%, range: 25% to 53%) and the average outbreak duration was 15 d (SD = 2 d, range: 6 to 26 d). In the high-risk base case scenario, the average clinical attack rate was 47% (SD = 23%, range: 22% to 100%) and the average outbreak duration was 15 d (SD = 2 d, range: 11 to 30 d).

Interventions

In the low-risk scenario, increasing the initial facility-level vaccine coverage resulted in a decreased clinical attack rate (Figure 2) and an outbreak of shorter duration. At a vaccine coverage of 93%, the average clinical attack rate decreased to 6% (SD = 4%, range: 1% to 16%) and the average duration of the outbreak decreased to 14 d (SD = 6 d, range: 6 to 33 d). Compared to the low-risk base case scenario, there was increased variability between simulation runs when horse-to-horse contact was reduced by 75%, which resulted in an average clinical attack rate of 34% (SD = 7%, range: 1% to 53%) (Figure 2). Reducing horse-to-horse contact also resulted in outbreaks of longer duration. When horse-to-horse contact was reduced by 75%, the average duration of the outbreak was 25 d (SD = 5 d, range: 6 to 53 d). The bundled interventions, which are described in

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Description</th>
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<tbody>
<tr>
<td>Increase initial vaccine coverage</td>
<td>Increased initial facility-level vaccine coverage from the base case value (63%) to 93% in 10% increments prior to the introduction of the infected horse.</td>
</tr>
<tr>
<td>Decrease horse-to-horse contact</td>
<td>Reduction in horse-to-horse contact by 25%, 50%, and 75% starting on the day following the introduction of the infected horse and continuing throughout the duration of the outbreak.</td>
</tr>
<tr>
<td>Bundled intervention</td>
<td>Combination of 73% initial facility-level vaccine coverage and a 25% reduction in horse-to-horse contact.</td>
</tr>
<tr>
<td>Bundled intervention</td>
<td>Combination of 73% initial facility-level vaccine coverage and a 75% reduction in horse-to-horse contact.</td>
</tr>
<tr>
<td>Bundled intervention</td>
<td>Combination of 83% initial facility-level vaccine coverage and a 25% reduction in horse-to-horse contact.</td>
</tr>
<tr>
<td>Bundled intervention</td>
<td>Combination of 83% initial facility-level vaccine coverage and a 75% reduction in horse-to-horse contact.</td>
</tr>
<tr>
<td>Bundled intervention</td>
<td>Combination of 93% initial facility-level vaccine coverage and a 75% reduction in horse-to-horse contact.</td>
</tr>
</tbody>
</table>

Table II, resulted in decreased variability between simulation runs, decreased average clinical attack rates, and outbreaks of shorter duration compared to either intervention alone. Bundle 6, which is a combination of 93% initial vaccine coverage and a 75% reduction in horse-to-horse contact, resulted in an average clinical attack rate of 3% (SD = 2%, range: 1% to 12%) and an average duration of outbreak of 9 d (SD = 4 d, range: 6 to 31 d).

The medium-risk scenario resulted in clinical attack rates that were highly variable between simulation runs (Figure 2). At a vaccine coverage of 93%, the average clinical attack rate was 7% (SD = 8%, range: 1% to 99%) and the average duration of the outbreak was 15 d (SD = 9 d, range: 6 to 127 d). When horse-to-horse contact was reduced by 75%, the resulting clinical attack rate decreased (mean = 34%, SD = 7%, range: 1% to 71%) and the duration of the outbreak increased (mean = 26 d, SD = 7 d, range: 6 to 100 d). Compared to the other interventions, bundle 6 resulted in the lowest clinical attack rate (mean = 3%, SD = 2%, range: 1% to 13%) and an outbreak with the shortest duration (mean = 9 d, SD = 4 d, range: 6 to 34 d).

Interventions applied in the high-risk scenario resulted in clinical attack rates with bimodal distributions (meaning that the expected
outbreaks could be small or could be much larger than expected) and increased variability between simulation runs (Figure 2). At a vaccine coverage of 93%, the clinical attack rate was lower than in the high-risk base case scenario (mean = 19%, SD = 34%, range: 1% to 100%) and the duration of the outbreak was shorter (mean = 14 d, SD = 6 d, range: 6 to 38 d). When horse-to-horse contact was reduced, there was no change in either the clinical attack rate (Figure 2) or the average duration of the outbreak (mean = 15 d, SD = 2 d, range: 12 to 31 d). Compared to the other bundled interventions, bundle 6 resulted in the lowest clinical attack rate (mean = 18%, SD = 32%, range: 1% to 100%) and the outbreak of shortest duration (mean = 14 d, SD = 6 d, range: 6 to 36 d). However, the average clinical attack rate in the high-risk scenario was greater than the average clinical attack rate in both the low- and medium-risk scenarios when bundle 6 was applied (18% in the high-risk scenario compared to 3% in the low- and medium-risk scenarios).

**Sensitivity analysis**

The results of the sensitivity analyses are presented in Table III. Varying the waning immunity distribution had the greatest impact on the average clinical attack rate in the medium- and high-risk scenarios. In the medium-risk scenario, immunity waning earlier than expected increased the average clinical attack rate to 45% (SD = 21%, range: 24% to 100%), but there was no change when immunity waned later than expected. In the high-risk scenario, the average clinical attack rate increased to 68% (SD = 32%, range: 24% to 100%) when immunity waned earlier and decreased to 38% (SD = 6%, range: 1% to 100%) when immunity waned later.

**Discussion**

This computer simulation study has demonstrated that implementing a combination of high facility-level vaccine coverage before an EI outbreak and a substantial reduction in horse-to-horse contact during an outbreak was the most effective strategy to minimize the impact of an EI outbreak in a facility. The results suggest that vaccination alone may be sufficient to minimize the extent of an outbreak, provided that horses in the facility have been recently vaccinated. As vaccine-induced immunity starts to wane (as observed in the medium- and high-risk scenarios), the clinical attack rate becomes less predictable as the variability between simulation runs increases.

In the high-risk scenario, all intervention strategies resulted in attack rates with bimodal distributions, which demonstrate the uncertainty of their effect in a population that has not been recently vaccinated. If horses in the facility have not been recently vaccinated, reducing horse-to-horse contact is most useful for slowing transmission (as implied by the outbreaks of longer duration), which subsequently provides more time to implement additional biosecurity and infection control measures. These results support the recommendation to use a combination of biosecurity and infection control measures for effective disease prevention and control.

In all scenarios, increasing the initial facility-level vaccine coverage had an important protective effect on EIV transmission. This finding supports previous modeling studies that have contributed to evidence-based recommendations for increased vaccine coverage in equine facilities (20,28). Although vaccination was effective in decreasing the average clinical attack rate in the medium- and high-risk scenarios, the variability between simulation runs increased as immunity waned over time. The effect of waning immunity during an EI outbreak has been previously described in field investigations in Ireland, in which horses that had been vaccinated 5 or more months before their exposure to EI became infected during a time when immunity would be expected (29). The results of the current study support the need for a safe, well-tolerated vaccine with long-lasting immunity, in addition to the use of vaccination strategies that shorten the period of time that horses in a population are susceptible.

As with any computer simulation study, our model makes several simplifying assumptions. There is currently little published data on...

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**Table III. Sensitivity analyses of the infectious period and the waning immunity distribution used in the agent-based model of an equine influenza outbreak in a simulated equine facility.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Average clinical attack rate % (SD)³⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low-risk</td>
</tr>
<tr>
<td>Infectious period a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>3.0 days</td>
<td>38 (6)</td>
</tr>
<tr>
<td>Maximum</td>
<td>7.0 days</td>
<td>38 (5)</td>
</tr>
<tr>
<td>Waning immunity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>Triangular distribution (min. 21 days; max. 150 days; mode 86 days)</td>
<td>38 (5)</td>
</tr>
<tr>
<td>Late</td>
<td>Triangular distribution (min. 81 days; max. 210 days; mode 146 days)</td>
<td>38 (5)</td>
</tr>
</tbody>
</table>

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a The infectious period was varied in increments of 0.5 between the minimum and maximum value. 

b Model-projected clinical attack rates only provided for minimum and maximum scenarios. 

c SD = standard deviation.
the average size of equine facilities in Canada. Unpublished data suggest that an average equine facility in Ontario, Canada boards 17 horses, but ranges from 1 horse to over 100 horses. Therefore, the population size of 100 horses used in this study might not be representative of all equine facilities, but would instead provide an overview of the impact of biosecurity and infection control measures in a larger equine facility. The model assumed that the vaccination schedule of horses in the facility was synchronized, which may not be representative of all facilities. If vaccination within a facility is not synchronized, the model scenarios might not accurately represent the time points at which horses will have decreased virological protection. Further research is warranted on the timing and frequency of vaccination in a facility to determine the appropriateness of the assumption of a synchronized vaccination schedule.

The assumption of a vaccine with 100% efficacy may be overly simplistic and provides conservative estimates for model projections. The use of this assumption would potentially underestimate the true attack rate in a facility using a less efficacious vaccine. Similarly, the assumption that vaccinated horses could not become infected or transmit infection would underestimate the expected attack rate in the absence of this assumption. Lastly, the distribution of waning immunity used in the model may vary depending on the type of vaccine product used and the individual immune status of the horse.

Future research is required to describe horse-to-horse contact patterns within a facility, including alternatives to the homogeneous mixing assumption. While our model was well-calibrated to observed outbreak data (23), each outbreak may have its own transmission dynamics, including the potential for indirect transmission of EIV (23,30). Calibration of our model to the observed outbreak data might have overestimated the contact rate if EIV was transmitted both directly and indirectly during the outbreak reported by Morton et al (23). Continued research on equine contact patterns would also provide insight into the equivalent situation in real life of reducing horse-to-horse contact by a certain percentage and its feasibility in Canadian equine facilities.

This study demonstrates the usefulness of computer simulation modeling in equine populations. Our findings support the practice of continued vaccination of horses with an effective vaccine to counteract potential gaps in protection due to waning immunity. In addition, the results provide evidence-based recommendations that promote a combination of vaccination and reduced contact as an effective infection control practice. This model could be used to communicate the benefits of sustained biosecurity practices to veterinary practitioners and horse owners. Lastly, this study highlights future research opportunities for using computer simulation models to inform the implementation of effective disease prevention and control measures in equine populations.

**Acknowledgments**

This work was supported by the Ontario Ministry of Agriculture, Food and Rural Affairs — University of Guelph (OMAFRA-U of G)
Research Partnership, Equine Guelph, and the Canada Research Chairs program. Graduate funding for Kelsey Spence was provided by an Ontario Graduate Scholarship and an Ontario Veterinary College Scholarship.

References

Comparison between cerebrospinal fluid and serum lactate concentrations in neurologic dogs with and without structural intracranial disease

Leontine Benedicenti, Giacomo Gianotti, Evelyn M. Galban

Abstract

The objectives of this study were to investigate the relationship between cerebrospinal fluid lactate and serum concentrations in dogs with clinical signs of central nervous system disease and to establish if cerebrospinal fluid lactate (CSF) concentrations are higher in dogs with structural intracranial disease (Group Pos-MRI) compared to dogs that have clinical signs of intracranial disease but no structural brain disease (Group Neg-MRI) based on magnetic resonance imaging (MRI) findings. Using a prospective study canine blood and cerebrospinal fluid were collected in 24 dogs with neurological signs after undergoing brain MRI. Dogs were divided in 2 groups. No significant difference between serum lactate (1.57 ± 0.9 mmol/L) and CSF lactate concentration (1.34 ± 0.3 mmol/L) was detected. There was a direct correlation between CSF and serum lactate concentration (R = 0.731; P = 0.01). No significant difference was found in CSF lactate concentration between the 2 groups of dogs (P = 0.13).

Résumé

Les objectifs de la présente étude étaient d’examiner la relation entre les concentrations de lactate du liquide céréphalo-rachidien (LCR) et du sérum chez des chiens présentant des signes cliniques de pathologie du système nerveux central et établir si les concentrations de lactate du LCR sont plus élevées chez les chiens avec une maladie intracrânienne structurale (Groupe Pos-MRI) comparativement à des chiens avec des signes cliniques de maladie intracrânienne mais sans maladie structurale du cerveau (Groupe Nég-MRI) sur la base des trouvailles en imagerie par résonnance magnétique (IRM). Utilisant une étude prospective, du sang canin et du LCR ont été prélevés chez 24 chiens avec des signes neurologiques après un examen par IRM du cerveau. Les chiens ont été séparés en deux groupes. Aucune différence significative ne fut détectée entre les concentrations de lactate sérique (1.57 ± 0.9 mmol/L) et de lactate du LCR (1.34 ± 0.3 mmol/L). Il y avait une corrélation directe entre les concentrations de lactate du LCR et du sérum (R = 0.731; P = 0.01). Aucune différence significative dans la concentration de lactate du LCR ne fut trouvée entre les deux groupes de chiens (P = 0.13).

(Traduit par Docteur Serge Messier)
Materials and methods

Twenty-four client-owned dogs with suspected intracranial disease presented to the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania for MRI of the brain and CSF analysis. Dogs with clinical or hematological signs related to cardiovascular, respiratory, renal, or liver disease, or any other detectable disease process not primarily located intracranially, were excluded from the study to avoid conditions that affect lactate metabolism or production. Dogs which experienced seizures in the 4 d leading up to the MRI were also excluded from the study given that seizure activity increases CSF lactate concentration for up to 3 d (6).

All patients underwent general anesthesia for brain MRI and CSF collection. Patients were divided into 2 groups based on imaging results. The first group had no evidence of structural brain disease (Group Neg-MRI) and the second group had evidence of structural brain disease (Group Pos-MRI). Anesthetic protocols varied but in most cases consisted of an opioid premedication [0.2 mg/kg body weight (BW) methadone or 0.1 to 0.3 mg/kg butorphanol, intravenously (IV)], followed by induction with propofol to effect (1 to 6 mg/kg BW, IV) (Diprivan 10 mg/mL; Fresenius Kabi USA, Lake Zurich, Illinois, USA), and maintenance with either isoflurane in 100% oxygen (Isothesia 250 mL; Henry Schein Animal Health, Dublin, Ohio, USA) or a propofol constant rate infusion (0.1 to 0.5 mg/kg BW per minute, IV). After a sagittal T2-weighted image of the brain was acquired, mannitol (Hospira; Lake Forest, Illinois, USA) or a propofol constant rate infusion (1 to 6 mg/kg BW, IV) (Diprivan 10 mg/mL; Fresenius Kabi USA, Lake Zurich, Illinois, USA), and maintenance with either isoflurane in 100% oxygen (Isothesia 250 mL; Henry Schein Animal Health, Dublin, Ohio, USA) or a propofol constant rate infusion (0.1 to 0.5 mg/kg BW per minute, IV). After a sagittal T2-weighted image of the brain was acquired, mannitol (Hospira; Lake Forest, Illinois, USA) or a propofol constant rate infusion (1 to 6 mg/kg BW, IV) was administered once there was evidence of a possible increase in intracranial pressure.

When the imaging study was complete, CSF and arterial blood samples were collected for lactate measurement. All CSF samples were collected from the cerebellomedullary cistern with the patient in lateral recumbency, following a standard technique (7). Blood was collected from an arterial catheter (BD Insyte; Becton Dickinson Infusion Therapy Systems, Sendy, Utah, USA) previously inserted in the dorsal pedal artery or the median sacral artery and placed in a red top blood collection tube (Monoject Blood Collection Tubes; Covidien, Mansfield, Massachusetts, USA). Blood samples were immediately centrifuged with a Horizon 642VES Centrifuge (Drucker Diagnostics, Port Matilda, Pennsylvania, USA) at 3200 rpm for 3 min to separate serum. Serum and CSF samples were analyzed by slide method on the VITROS 350 System (Ortho Clinical Diagnostics, Raritan, New Jersey, USA) at the time of collection. Quality control was run daily for lactate on the Vitos 350 and results had to be in range before patient samples were run with the analyzer.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Breed</th>
<th>TNCC (μL)</th>
<th>TP (mg/dL)</th>
<th>RBC (μL)</th>
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<td>37</td>
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</table>

TNCC — Total nucleated cell count; TP — Total protein; RBC — Red blood cell concentration.
Statistical analysis

Statistical analysis was performed using XLSTAT (Addinsoft, New York, New York, USA), a standard data analysis software.

A Shapiro-Wilk test was conducted to determine if CSF and serum lactate concentrations followed a normal distribution. The correlation between CSF lactate and serum lactate concentrations was tested with Pearson's correlation test. The CSF and serum lactate concentrations in all dogs and between the 2 groups were tested using a two-tailed t-test. All data are presented as mean ± standard deviation (± SD). Statistical significance was set at $P < 0.05$.

Results

A total of 24 dogs were enrolled in the study. They presented with a wide spectrum of clinical signs, including: seizures in 10 dogs (42%), signs referable to vestibular neurolocalization in 5 dogs (21%), upper motor neuron/general proprioceptive ataxia and circling in 2 dogs (8%), multifocal intracranial signs in 2 dogs (8%), altered mentation in 1 dog (4%), unilateral facial paralysis and Horner’s syndrome in 1 dog (4%), neck pain in 1 dog (4%), and restless and difficulty with mastication in 1 dog (4%). Breeds included 4 mixed breed dogs, 2 Labrador retrievers, 3 golden retrievers, 2 Weimaraners, and 1 each of the following breeds: cocker spaniel, bichon frise, Pembroke Welsh corgi, American pit bull, English bulldog, pug, Brittany spaniel, boxer, bloodhound, toy Manchester terrier, German shepherd, Maltese, and Plott hound. Twelve dogs were neutered males, 11 were spayed females, and 1 was an intact male. Disease distribution was as follows: 11 dogs (46%) had normal MRI results (Group Neg-MRI); of these, 8 (73%) were diagnosed with idiopathic epilepsy, 1 (9%) with idiopathic vestibular disease, 1 (9%) with unilateral vestibulocochlear nerve dysfunction of unknown origin, and 1 (9%) with altered mentation. Thirteen dogs (54%) had structural changes on MRI (Group Pos-MRI). In this group, the lesions were consistent with suspect neoplasia in 5 dogs (38%), cerebrovascular accidents in 4 dogs (31%), meningoencephalitis of unknown etiology in 2 dogs (15%), otitis interna with secondary meningitis in 1 dog (8%), and congenital malformation (cerebellomedullary cyst) in 1 dog (8%). Data on the individual patient characteristics including breed, routine CSF results, blood contamination, and definitive diagnosis are shown in Table I. Since xanthochromic fluid has a high lactate concentration irrespective of diagnosis (8), it was excluded from the study. Recent blood contamination has not been reported to affect lactate concentration (9).

Two dogs received mannitol IV over 15 min. Cerebrospinal fluid collection was completed less than 60 min after starting infusion of the osmotic agent. Mannitol has been reported to increase lactate concentration in arterial blood and CSF over time, specifically at 360 min after administration (10). Since our samples were collected less than 60 min after starting mannitol infusion, we do not feel that lactate concentration was affected by the osmotic agent.

No significant difference between serum lactate (1.57 ± 0.9 mmol/L) and CSF lactate (1.34 ± 0.3 mmol/L) concentration was detected (Figure 1). Furthermore, there was a direct correlation between CSF lactate concentrations and serum lactate concentrations ($R = 0.731, P = 0.001$).

When data were analyzed comparing group Neg-MRI to group Pos-MRI, no significant difference was found in CSF lactate concentrations (1.2 ± 0.2 mmol/L and 1.4 ± 0.4 mmol/L, respectively; $P = 0.13$).

Discussion

The first objective of this study was to evaluate the relationship between CSF and serum lactate concentrations in dogs with clinical signs of CNS disease. There was no statistical difference between CSF lactate concentrations and serum lactate concentration in all dogs and among groups. There was, however, a direct correlation between
CSF and serum lactate concentrations in all dogs. Our results are in
direct contrast with the existing literature, as independence of blood
and CSF lactate concentration has been previously demonstrated in
humans and animal models (11). Because lactic acid is fully ionized
at physiological pH levels, it has been postulated that it does not
diffuse rapidly across the blood-brain barrier. Using a dog model
in an experimental setting, it has been demonstrated that raising
the arterial lactate concentration by IV infusion of lactate does not
significantly alter the CSF lactate concentration (12). Observation
in a clinical setting reported that humans suffering from a coma as
result of a structural brain disease had CSF lactate concentrations
4 to 8 mEq/L higher than lactate concentrations measured in either
venous or arterial blood (11). A possible explanation for our results
being in contrast with the current literature could be that none of
the dogs across both groups had higher CSF lactate concentrations
compared to their respective serum values.

In the present study, the serum lactate concentration was evalu-
ated in arterial samples only. The feasibility of using peripheral
venous lactate measurements as a reliable substitute for arterial
lactate measurement has been investigated (13,14). In veterinary
medicine, the agreement between arterial and peripheral venous
lactate concentrations has been evaluated in healthy dogs with the
conclusion that the discrepancy between sample sites is clinically
insignificant (14). However, Gallagher et al (13) reported that despite
the high correlation between arterial and venous lactate measure-
ments, arterial lactate measurements are not an accurate and reli-
able replacement for venous lactate measurements. Since the use of
venous lactate values as surrogates for arterial lactate levels has been
cautions, it was decided to only measure the lactate concentration
in arterial samples in our study.

Additionally, dogs with an altered metabolic status that could
have been responsible for changes in peripheral blood lactate
concentration were not enrolled in this study. Therefore, dogs with
coeexisting diseases such as respiratory or kidney disease that might
have been responsible for altered serum lactate concentrations did
not meet our inclusion criteria.

The second objective of this study was to establish if CSF lactate
concentrations were higher in dogs with structural intracranial dis-
 ease than in dogs with clinical signs of intracranial disease but no
structural signs according to MRI. Our hypothesis was supported
by previous research that evaluated CSF lactate concentration in
different disease processes involving changes within the intracranial
space. A prospective study by Caines et al (5) evaluated CSF lactate
concentrations in healthy dogs and dogs with a neurological dis-
ease undergoing general anesthesia for MRI and observed a trend
whereby higher concentrations of CSF lactate were detected in canine
cases with worse neurological scores. In the present study design,
authors included all clinical cases presenting with neurological signs
to investigate the possibility of CSF lactate values being higher in
dogs with confirmed structural intracranial disease. The confirma-
tion of a statistically significant difference between these 2 groups
would have been a very helpful and inexpensive diagnostic screen-
ing tool able to orient the clinician towards a potential diagnosis of
intracranial disease. However, the results of our study do not suggest
that dogs with structural intracranial disease have higher CSF lactate
concentrations compared with serum values.

Lactate levels have been evaluated in the CSF of humans with
various neurological conditions. Status epilepticus has been associ-
ated with a significant increase in CSF lactate and the concentration
value can serve as a predictive indicator of morbidity and mortality
(15). Severe brain injury following trauma has been associated with
increased CSF lactate levels immediately following the impact in cat
models (16). In humans, serial assessments of CSF lactate concen-
trations following head injury have been used as an indicator of pro-
gnosis and clinical course during hospitalization (17). Increased CSF
lactate concentrations have been reported in patients with bipolar
disorder and schizophrenia as a result of impaired mitochondrial
metabolism (18).

Extensive investigation of CSF lactate levels has been conducted
in humans with bacterial and viral meningitis. A correlation between
elevated lactate levels has been reported with the presence of
bacterial meningitis as well as viral infections of the CNS (19–21).
Cerebrospinal fluid lactate concentration is considered a good single
indicator to distinguish bacterial meningitis from aseptic meningitis
in humans (22). The D-lactate isomer of lactic acid is a specific
metabolite found in lower forms of life (23); bacteria produce it but
mammalian cells cannot. When D-lactic concentrations were mea-
sured in CSF of humans with suspected bacterial meningitis, a higher
sensitivity value than the one reported by Gram stain technique was
detected (24). In the present study, the authors elected to measure
L-lactic acid levels only, given the infrequency of bacterial meningitis
in the canine population.

Finally, CSF lactate levels increased in canine experimental models
of iatrogenic intracerebral hematoma or subarachnoid hemorrhage
(4). In that study, the increase in CSF lactate was significantly
greater in dogs with intracerebral hematoma than in dogs with subarachnoid
hemorrhage. The authors suggested that the rise in lactate derived
not only from shed blood cells but also reflected the anaerobic state
of the brain. They indicated that the increase in CSF lactate concen-
trations could be used as an indicator for the severity of cerebral
hypoxia or the prognosis of patients with intracerebral hematoma (4).
In our study population, 4/19 dogs were diagnosed with vascular
events consistent with ischemic (non-hemorrhagic) infarcts. None of
these patients had increased CSF lactate concentrations compared
with serum values.

One of the dogs in the study herein presented with altered men-
tation along with mild neck pain. The dog was a 12-year-old male
neutered toy Manchester terrier with no structural disease evident
in the MRI. Given the age of the dog, canine senile dementia could
be considered as a differential diagnosis for the altered mentation.
A previous study evaluating the cognitive status of 25 dogs through
a rapid behavioral test demonstrated a parallel increase of CSF
lactate, pyruvate, and potassium concentrations in the dogs with
severe cognitive deficits (25). The CSF lactate concentrations did not
significantly differ between the patient with suspected age-related
changes in mentation and the other dogs enrolled in this study.

The potential limitations of the study reported here are the rela-
tively small size of the groups and the lack of definitive diagnoses
via histopathology for cases with confirmed intracranial structural
diseases. A multicenter approach could be considered to increase
the number of dogs available for evaluation with storage of the CSF
samples for later analysis. Obtaining a definitive diagnosis for the
dogs with detectable intracranial lesions would allow for the evaluation of different groups of patients based on the disease process affecting them. It is possible that a relationship between CSF lactate concentration values and a specific disease process has been missed in the present study due to the few cases evaluated.

The ability to infer a reliable diagnosis of intracranial disease from CSF lactate concentration is an attractive prospect; the present research could not demonstrate a significant increase in CSF lactate concentration in dogs with intracranial disease compared to serum values. Routine measurements of lactate concentration in CSF are therefore not recommended in a clinical setting.

References

Roles of the crp and sipB genes of Salmonella enterica serovar Typhimurium in protective efficacy and immune responses to vaccination in mice

Songbiao Chen, Chengshui Liao, Chunjie Zhang, Xiangchao Cheng

Abstract

Salmonella enterica serovar Typhimurium has a wide host range and is capable of causing infections ranging from severe gastroenteritis to systemic infection in humans. To determine if attenuated S. Typhimurium strains can serve as safe and effective oral vaccines to prevent typhoid fever, the biologic characteristics of crp and sipB deletion mutants were evaluated. Previous studies had found that the crp and sipB genes are related to Salmonella pathogenicity. In this study, cytotoxicity, protective efficacy, and immune responses of the host were analyzed. Our previous data had shown a significance decrease in virulence for the crp and sipB mutants compared with a wild-type strain. The current study confirmed this finding in HeLa cells and showed that the crp mutant was significantly less cytotoxic (P < 0.05) than the sipB mutant. Mice vaccinated with the crp mutant showed significantly better protection after challenge with the wild-type strain (P < 0.05) and significantly greater responses in serum IgG (P < 0.01) and secretory IgA (P < 0.05) compared with the mice vaccinated with the sipB mutant (P < 0.05). Our results indicate that the crp mutant has the potential to be a vaccine candidate and is safe in mice.

Résumé

Salmonella enterica sérovar Typhimurium a un large spectre d’hôte et est capable de causer chez l’humain des infections allant d’une gastroentérite sévère à une infection systémique. Afin de déterminer si des souches atténuées de S. Typhimurium pourraient servir de vaccin oral sécuritaire et efficace afin de prévenir la fièvre typhoïde, les caractéristiques biologiques de mutants ayant subis des délétions dans les gènes crp et sipB furent évalués. Des études antérieures ont démontré que les gènes crp et sipB sont associés à la pathogénicité de Salmonella. Dans la présente étude, la cytotoxicité, l’efficacité protectrice, et les réponses immunitaires de l’hôte ont été analysées. Nos données antérieures avaient démontré une diminution significative de la virulence pour tous les mutants crp et sipB comparativement à une souche sauvage. Cette étude a confirmé ces données chez les cellules HeLa et démontré que le mutant crp était significativement moins cytotoxique (P < 0.05) que le mutant sipB. Après une infection défi avec une souche sauvage, les souris vaccinées avec le mutant crp étaient significativement mieux protégées (P < 0.05) et présentaient une plus grande réponse en IgG sérique (P < 0.01) et IgA sécrétoire (P < 0.05) comparativement aux souris vaccinées avec le mutant sipB. Nos résultats indiquent que le mutant crp possède le potentiel pour être un vaccin candidat et est sécuritaire chez la souris.

(Traduit par Docteur Serge Messier)

Introduction

Salmonella enterica sérovar Typhimurium has a wide host range and can cause infections in humans ranging from severe gastroenteritis to systemic infection (1). Vaccination is an effective method for preventing Salmonella infections (2,3). Attenuated live vaccines provide protective immunity through activation of both antibody and cell-mediated immune responses (2,4,5). In many studies, deletion mutants have been reported to provide protective immunity. For example, the S. Typhimurium attenuated strain SR-11 deficient in phoP or aroA can provide protection against oral challenge with $5 \times 10^8$ colony-forming units (CFU) of the virulent strain of S. Typhimurium (6). In addition, the NalR-RifR Rtt deletion strain of S. Typhimurium can help to control Salmonella 4,12:i:- infections in poultry (7). Curtiss et al (8) described means of achieving regulated delayed attenuation of S. Typhimurium strains in vivo.

Salmonella invasion protein B (SipB) is the starting point of the invasion process; sipB is one of the genes encoding the Salmonella type 3 secretion system transport effector proteins. Out of an array of Salmonella pathogenicity island 1 (SPI-1) effector proteins, Sip effector are well-studied and are known to play an important role in Salmonella invasion of nonphagocytic cells and the triggering of gut inflammation (9,10). SipB can bind to SipC through its C-terminal domain, which facilitates membrane insertion and subsequent translocation in the host cell membrane (11). SipB can induce cell death via the adapter proteins Tram and Trif (12) and plays an essential role in
Salmonella pathogenesis (13). The crp gene, conserved in diverse bacterial species, encodes the cyclic adenosine monophosphate (cAMP)-activated receptor protein (CRP), a global regulator that modulates expression of a number of genes required for virulence, carbohydrate metabolism, flagellum synthesis, and glycogen synthesis (14,15).

Previous studies have constructed sipB and crp deletion mutants through a double-crossover event with use of the suicide vector pRE112 (16). The deletions were verified by DNA sequencing of the products of polymerase chain reaction. The results showed that sipB and crp were related to virulence (16). However, no studies have examined the potential of using a sipB or crp deletion mutant to prevent S. Typhimurium infection. Our previous data showed a significant decrease in the virulence of sipB and crp mutants compared with a wild-type S. Typhimurium strain (16). In the current study we further evaluate the protective efficacy of crp and sipB deletion mutants of S. Typhimurium as live attenuated candidate vaccines.

**Materials and methods**

**Bacterial strains and cell lines**

We used S. Typhimurium strain SL1344 and its isogenic sipB and crp mutants in this study, as in our previous work (16). HeLa cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and incubated at 37°C with 5% CO₂.

**Cytotoxicity assay**

This assay, done as described, used an MTT cell proliferation and cytotoxicity assay kit by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, HeNan, China). Briefly, 10⁴ HeLa cells were seeded per well in a 96-well plate and incubated overnight at 37°C with 5% CO₂. The cells were infected the same multiplicity of infection (MOI) of S. Typhimurium strain SL1344 or an isogenic sipB or crp mutant MOI of 100:1 (each test was repeated at four times per 4 wells), at the same time, an uninfected group was established as a control group. After incubation for 2 h at 37°C with 5% CO₂, the supernatant was removed, and 50 μL of 1 x 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was added to each well; the cells were further incubated for 4 h at 37°C with 5% CO₂. The supernatant was removed and 150 μL of dimethylsulfoxide added. The optical densities (ODs) at 570 nm were recorded.

**Immune protection experiment**

This experiment was carried out as previously described (17). Four groups of 40 (N = 160) specific-pathogen-free BALB/c mice 6 wk of age, obtained from the experimental animal center of Zhengzhou University, Zhengzhou, Henan province, China, were infected with S. Typhimurium SL1344 and the isogenic mutants, 10 mice per strain. From the results of a pilot study, each mouse receiving a mutant strain was inoculated orally with 1.0 x 10⁹ organisms in 0.2 mL of phosphate-buffered saline (PBS), and the positive and negative control groups were inoculated orally with 0.2 mL of PBS. The mice in the mutant and the control groups were challenged 4 wk after vaccination with 4 x 10⁷ CFU of S. Typhimurium SL1344 in 0.2 mL of PBS. The mortality rate was calculated using 40 mice for 30 d after challenge. The IgG level was measured using 30 mice and 90 mice were used for the SlgA test.

The serum IgG concentration was determined weekly after vaccination by enzyme-linked immunosorbent assay (ELISA) as described previously (6,17). The blood samples were collected from the caudal vein. Heat-killed S. Typhimurium SL1344 was used as the coating antigen (30 μg/mL, 100 μL/well). After incubation overnight at 4°C and then incubation with skimmed milk for 1 h, samples were washed 3 times with 200 μL of PBS and 0.05% Tween (PBS-T) per well. Next, 100 μL of a 1:200 dilution of the serum to be tested was added and the mixture incubated at 37°C for 60 min and then washed 3 times with 200 μL of PBS-T per well. Rabbit IgG against mouse antigen, labelled with horseradish peroxidase, was added, 100 μL per well, and the mixture incubated at 37°C for 60 min. The chromogenic substrate 3,3′,5,5′-tetramethylbenzidine was then added and the OD450 determined with an ELISA reader after the reaction was stopped with 2 mol/L of H₂SO₄.

For weekly determination of the secretory IgA (slgA) antibody response after vaccination, mice were killed and the slgA levels in intestinal samples assayed used Mouse Secretory slgA detection kit by ELISA (ShangHai Shifeng Bioengineering Institute, ShangHai, China).
China) each week for 5 wk. In accordance with the manufacturer’s instructions, the full intestine was collected minus excess fat and the pancreas, washed with PBS buffer containing 1 mmol/mL of the protease inhibitor phenylmethylsulfonyl fluoride, slit with scissors, and cut into 1-cm-diameter pieces, which were ground in a mill and then centrifuged at 8000 \times g at 4°C for 15 min. Samples were collected from the vaccinated mice as well as intestinal content controls.

**Statistical analysis**

All data were expressed as the mean ± standard error of the mean unless otherwise specified and analyzed with GraphPad Prism (GraphPad Software; San Diego, California, USA). The data were analyzed by 1-way analysis of variance (ANOVA) and Student’s *t*-test. A *P*-value < 0.05 was considered significant.

**Results**

In the cytotoxicity assay, after 6 h of infection of the HeLa cells, virulence was significantly greater in the group infected with wild-type *S. Typhimurium* SL1344 compared with the control group and the mutant groups. Virulence was significantly lower in the *crp* mutant group compared with the *sipB* mutant group.

To examine the protective efficacy of the *crp* and *sipB* mutant strains, mice were orally challenged with 4 \times 10^7 CFU of the wild-type strain of *S. Typhimurium* 4 wk after vaccination. Over the next 30 d each of the 3 vaccinated groups had a significantly lower mortality rate (*P* < 0.05) compared with the parent strain, and the *crp* deletion mutant was more attenuated than the *sipB* deletion mutant. These results are consistent with the work of Santos (18), who reported that *S. Typhimurium* induces cell death in bovine macrophages by 2 distinct mechanisms: early *SipB*-mediated and delayed *SipB*-independent. In addition, Cook et al (12) reported that *Salmonella*-induced *SipB*-independent cell death requires toll-like receptor-4 signalling *via* the adapter proteins Tram and Trif. In more recent studies the effects of *crp* deletion on the phenotype, virulence, and immunogenicity of avian *Pasteurella multocida* and *Edwardsiella ictaluri* were investigated (19,20). Ou et al (21) reported that CRP is related to biofilm formation, fimbria production, capsular polysaccharide biosynthesis, and lethality of *Klebsiella pneumoniae* serotype K1 for mice, causing pyogenic liver abscess. In addition, it can influence bacterial virulence and transcriptional regulation of allS in *Klebsiella pneumoniae* (22), which is consistent with our observations. These findings show that the attenuated *crp* mutant may influence biofilm formation and fimbria production, and the attenuated *sipB* mutant may influence macrophage cell death.

It is very important that live attenuated *Salmonella* vaccines have protective efficacy against wild-type *S. Typhimurium* (2). Therefore, we also evaluated the protective efficacy of the *crp* and *sipB* mutant strains against oral challenge with SL1344 by determining mice survival rates after vaccination. The vaccinated groups had a significantly lower mortality rate (*P* < 0.05) than the control challenged group. The mortality rates for the 2 groups vaccinated with the mutants differed significantly (*P* < 0.05), at 10% for the group vaccinated with the *crp* mutant and 40% for the group vaccinated with the *sipB* mutant. In addition, specific humoral and mucosal immune responses induced by live attenuated *Salmonella* vaccines are crucial for the natural host (2). In this study we found a strong specific serum IgG response in the mice vaccinated with the mutant candidates, greater for the *crp* mutant than for the *sipB* mutant, and the antibodies were detectable by indirect ELISA at 1 wk. The antibody levels were significantly greater than in the nonvaccinated mice. A significantly elevated slgA response, greater for the *crp* mutant than for the *sipB* mutant, was clearly observed after vaccination.

**Discussion**

In this study we evaluated the efficacy of highly attenuated *crp* and *sipB* mutants as live vaccines against salmonellosis in the murine typhoid model. We used strains of *S. Typhimurium* SL1344 deficient in *crp* and *sipB* and investigated their cytotoxicity and protective efficacy as well as the immune responses evoked in mice.

In our previous study we had determined that the virulence of these mutants in mice was significantly reduced from that of the wild-type *S. Typhimurium* (16). Therefore, we further investigated the effect of *Δcrp* and *ΔsipB* on cytotoxicity in HeLa cells *in vitro*. We determined that the *Δcrp* and *ΔsipB* deletion mutants were attenuated in comparison with the parent strain, and the *Δcrp* deletion mutant was more attenuated than the *ΔsipB* deletion mutant. These results are consistent with the work of Santos (18), who reported that *S. Typhimurium* induces cell death in bovine macrophages by 2 distinct mechanisms: early *SipB*-mediated and delayed *SipB*-independent. In addition, Cook et al (12) reported that *Salmonella*-induced *SipB*-independent cell death requires toll-like receptor-4 signalling *via* the adapter proteins Tram and Trif. In more recent studies the effects of *crp* deletion on the phenotype, virulence, and immunogenicity of avian *Pasteurella multocida* and *Edwardsiella ictaluri* were investigated (19,20). Ou et al (21) reported that CRP is related to biofilm formation, fimbria production, capsular polysaccharide biosynthesis, and lethality of *Klebsiella pneumoniae* serotype K1 for mice, causing pyogenic liver abscess. In addition, it can influence bacterial virulence and transcriptional regulation of allS in *Klebsiella pneumoniae* (22), which is consistent with our observations. These findings show that the attenuated *crp* mutant may influence biofilm formation and fimbria production, and the attenuated *sipB* mutant may influence macrophage cell death.

Mucosal slgA antibodies were efficiently induced by oral vaccination (Figure 3). The groups vaccinated with the mutants showed a significantly greater response each week beyond week 1 compared with the control group (*P* < 0.05). The *Δcrp* group showed a significantly greater response than the *ΔsipB* group only at weeks 4 and 5 (*P* < 0.05).
however, the levels began to decrease at 4 wk, which may be related to bacterial colonization and persistence in internal organs (23,24).

In summary, our present work demonstrates that the S. Typhimurium Δcrp deletion mutant, when given orally in a single dose, can induce a protective immune response upon subsequent challenge with virulent *Salmonella*. In addition to its use as a live attenuated vaccine, the *crp* deletion mutant might be used as a heterologous antigen delivery system, a very attractive option that to date remains unexplored.

**Acknowledgments**

This study was supported by grants from the National Natural Science Foundation of China (grant 31572489), the PhD Start-up Fund of Henan University of Science and Technology (grant 13480071), and the Key Project of Henan Province for Scientific Research and Higher Education of China Colleges and Universities (grant 17A230009).

**Conflict of interest**

The authors or their institutions do not have any relationships that may influence or bias the results and data presented in this manuscript.

**References**


Venous blood gases, plasma biochemistry, and hematology of wild-caught common chameleons (Chamaeleo chamaeleon)

David Eshar, Melanie Ammersbach, Boaz Shacham, Gad Katzir, Hugues Beaufrère

Abstract

The purpose of this study was to determine a wide range of selected hematologic, venous blood gases, and plasma biochemistry analytes in common chameleons (Chamaeleo chamaeleon). Blood samples were collected from the ventral tail vein of 41 common chameleons to determine reference intervals for 30 different blood analytes. The calcium-to-phosphorus ratio, packed cell volume (PCV), refractometric total solids (TS), blood cell counts, and differentials were also determined. The microscopic evaluation of blood smears revealed inclusion bodies in monocytes in 7 of the samples. Females showed significantly higher values of plasma proteins and calcium and cholesterol concentrations and males showed significantly higher values of aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) plasma concentrations. Significant differences were found between similar analytes determined by different testing methodologies in the PCV/hematocrit, electrolytes (sodium, potassium), and plasma proteins (TS, total protein (TP) and albumin). Blood analytes determined in this study can provide baseline data that may be useful when evaluating the health status of common chameleons, taking into consideration the potential effects of gender and the type of analyzer used.

Introduction

The common chameleon (Chamaeleo chamaeleon), also known as the Mediterranean chameleon, is a medium-sized, arboreal lizard species that inhabits natural forests and plantations in the Mediterranean zone and is widely distributed in southern Europe, northern Africa, and southwestern Asia (1). Chameleons are slow-moving, diurnal lizards, and in Israel, they are mostly active during the warm months (May to November) (2).

Reptile medicine and research relies on laboratory analyses to evaluate health status, but reference ranges for most hematology, biochemistry, and other physiological variables of common chameleons are limited (3,4). Only a few studies of a small number of common chameleons (n < 11) were found to report selected hematology and blood biochemistry parameters (3–5). Physiologic parameters, such as electrolytes, enzymes, metabolites, and proteins, are important factors that can be used to assess metabolic and homeostatic disturbances, tissue perfusion, and the overall health status of the animal (6,7). The number of parameters that can be analyzed at any given time, however, is limited by the small body size, low total blood volume (5% to 8% of total body weight in reptiles), and challenging venous access that allows only a small blood sample to be collected (7). Point-of-care (POC) analyzers may allow rapid decisions to be made in critical patients and provide field researchers and zoological veterinarians with prompt results while using a relatively small volume of blood (7–11). After...
Materials and methods

Animals

Common chameleons sampled in this study were wild-caught from the central Mediterranean coastal region of Israel (roughly between 32°N–33°N and just west to 35°E). Blood sampling for this study was done during the summer months of June and July when the average day/night temperatures were 32°C/25°C and the mean relative humidity 70%. The chameleons were judged to be healthy following a complete physical examination that showed no overt signs of disease. For each chameleon, the weight was accurately obtained using a digital gram scale and the snout-to-vent length (SVL) was accurately measured to the nearest 1 mm using a ruler. Although the age could not be accurately determined in these wild chameleons, only animals with an SVL of > 90 mm were sampled in this study and were estimated to be mature and at least 1 y old (14). The gender was determined by observing the bulges of the 2 hemipenes at the base of the tail in mature males and the smooth taper from the vent area towards the tail in females. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC#3407) of the College of Veterinary Medicine at the Kansas State University and under a permit from the Israeli Nature and Parks Authority (2014/40513).

Analyzers

The VetScan VS2 Analyzer (Abaxis Veterinary Diagnostics, Union City, California, USA) is a veterinary bench-top analyzer that is marketed for use in reptiles (12). The small analyzer (15 × 32 × 20 cm, 5.1 kg) uses plastic single-use rotors containing dry reagent beads configured for either specific health profiles (kidney, liver, comprehensive, other) or species (equine, canine, large animal, avian/reptile) and requires 100 µL of whole blood, serum, or plasma for the analysis. The rotor designed for birds and reptiles (VetScan Avian/Reptilian Profile Plus (VSARP); Abaxis Veterinary Diagnostics) measures 11 analytes and calculates 1 value [Globulins (Glob)] within 15 min. The VSARP provides concentrations of aspartate aminotransferase (AST, U/L), bile acids (BA, µmol/L), creatine kinase (CK, U/L), uric acid (UA, mg/dL), glucose (Glucose, mg/dL), total calcium (Ca, mg/dL), phosphorus (P, mg/dL), total protein (TP, g/dL), albumin (Alb, g/dL), globulins (Glob, g/dL), potassium (K, mmol/L), and sodium (Na, mmol/L).

The VetScan Mammalian Liver Profile (VSMLP) reagent rotor (Abaxis Veterinary Diagnostics) was also used in this study as it measures several biochemistry analytes that are not included in the VSARP. The VSMLP measures 8 analytes, including alanine aminotransferase (ALT, U/L), albumin (Alb, g/dL), alkaline phosphatase (ALP, U/L), bile acids (BA, µmol/L), total bilirubin (Tbil, mg/dL), cholesterol (Chol, mg/dL), gamma-glutamyl transferase (GGT, U/L), and blood urea nitrogen (BUN, mg/dL).

The iSTAT system (Abaxis Veterinary Diagnostics) consists of 2 main components: a handheld, battery-operated analyzer and disposable cartridges with micro-fabricated biosensors (15). This assay requires only 95 µL of heparinized whole blood and the analysis is completed within approximately 2 min, which is an advantage when testing blood samples in small-bodied chameleons with limited blood volumes (7,15,16). This analyzer was chosen for this study as it was advocated for use in wildlife field studies in general and specifically in reptiles (7,11,16–21). The specific iSTAT cartridge used in this study [CG8+ cartridges (iSTCG8), Abbott Laboratories, Abbott Park, Illinois, USA] can provide multiple parameters, including the hematocrit (Hct, %), hemoglobin (Hb, g/dL), blood pH, partial pressure of carbon dioxide (PaCO₂), partial pressure of oxygen (PaO₂), base excess (BE), bicarbonate (HCO₃−), total carbon dioxide content (TCO₂), saturation of oxygen (SO₂), potassium (K, mmol/L), sodium (Na, mmol/L), ionized calcium (iCa, mmol/L), and glucose (Glucose, mg/dL).

Blood sample collection and analysis

The chameleons were manually restrained and blood samples were collected aseptically from the ventral tail vein. A venous blood sample was collected using a 25–27G 1/2 to 5/8” hypodermic needle attached to a 1-mL syringe (BD SafetyGlide Syringe, Becton Dickinson, Franklin Lakes, New Jersey, USA) and placed in a 0.5-mL lithium-heparin collection tubes (BD Microtainer; Becton Dickinson).

All blood gases and biochemistry analyses were carried out immediately. The basic biochemistry profile was done on all of the blood samples using the VSARP and the calcium-to-phosphorus ratio (Ca:P) was later calculated for each chameleon. For each blood gas or biochemistry analysis, a 100-µL aliquot of blood was placed into a VetScan rotor or an iSTAT cartridge. Each rotor or cartridge was used within 10 min of removing it from refrigeration and immediately after opening its protective pouch. Tests were then analyzed immediately after the rotor or cartridge was filled. Before this study, the VetScan VS2 and the iSTAT analyzers were functioning without a problem on a routine basis and their software was updated regularly according to the manufacturer’s instructions. All samples were run by the same operator.

The PCV was determined routinely by the microhematocrit method in heparinized capillary tubes and centrifuged at 12 000 × g for 5 min and the total solids (TS) were read from the spun and broken tube using a handheld, temperature-compensated refractometer. Blood smears were prepared using a squashe preparation and were air dried until later staining using an automated stainer (Aerospray Stainer; Wescor, Logan, Utah, USA) with a modified Wrights stain (Wescor Cytology Reagents). The total white blood cell count (TWBC) was estimated from the smear by counting 10 fields at 400× magnification. The microscope-calibrated formula used was: TWBC (in cells × 10³/L) = average WBC per 400× × 1.5. The blood smears were examined in order to conduct a 100-leukocyte differential, used to calculate absolute values for each cell type and to
assess the morphology of all cells. The hematopoietic profile included
WBC and rubricytes count and heterophils, lymphocytes, monocytes,
azurophils, eosinophils, and basophils (counts and %).

**Data analysis**

Reference intervals were determined according to the ASVCP
and Clinical and Laboratory Standards Institute (CLSI) guidelines
(22,23). Because of the smaller sample size (< 120), the reference
limits were calculated using a robust approach, both when normally
distributed and after Box-Cox transformation when not normally
distributed (23,24). Ninety percent confidence intervals (CI) of
the reference limits were obtained using a bootstrap approach to
assess precision of the reference interval limits as recommended.
Outliers were detected using Tukey and Dixon methods and
removed accordingly. Only the median and range were reported for
10 ≤ n < 20.

Values were compared between genders using 2-sided, 2-sample
t-tests for normally distributed variables with homogeneous varia-
ces and 2-sample Wilcoxon tests for other variables. Values were
compared between monocyte inclusion body positive and negative
animals using 2-sample Wilcoxon tests for other variables.

The agreement of selected pairs of variables (TS and VetScan TP,
PCV and iSTAT hematocrit, albumin VSARP versus VSMLP, VetScan
versus iSTAT for K, Na, and glucose) was investigated using Passing-
Bablok regression analysis (25). The constant bias is represented by the
intercept of the regression line and should be different from 0
to be significant (0 not included in the 95% CI), whereas the pro-
portional bias is represented by the slope of the regression line and
should be different from 1 to be significant (1 not included in the
95% confidence interval). A cumulative sum of residuals (CUSUM)
test of linearity was carried out. The 95% limits of agreement around
the mean bias were obtained by the following formula:

\[
\text{bias} = 1.96 \sqrt{\frac{s^2}{n}}
\]

with \(s^2\) the variance of the bias. Clinical allowable error limits were
defined as 10% (26). Since globulins were a calculated value for the
VetScan, agreement statistics were not done on this analyte.

Graphs were produced with one method as the x-axis, the second
method as the y-axis, the biochemistry data points, the equality
line, the clinical allowable error limits around the equality line, the
Passing-Bablok regression line (mean bias), and the 95% limits of
agreement around the mean bias. The more disagreement there was,
the more divergent the Passing-Bablok regression line (bias line) was
from the equality line. For clinically acceptable agreement, 95% of the
data points (or the limits of agreement) should be contained within
the clinical allowable error limits. Spearman correlation coefficients
were also obtained.

When not specified otherwise, an alpha of 0.05 was used for
statistical significance. The R software [R development core team
(2012), R foundation for statistical computing, Vienna, Austria.
http://www.r-project.org] was used for statistical analysis with the
R-package “MethComp” [MethComp: functions for analysis of agree-
http://cran.r-project.org/package=MethComp]. The R package was
used for Passing-Bablok regression analysis. Reference values were
determined using Reference Value Advisor (27).

![Image](https://example.com/image.png)

**Results**

The study included 41 chameleons (18 females and 23 males). There
were no differences in weight (\(P = 0.72\)) or length (\(P = 0.79\)) between
male and female chameleons in this study, showing (mean ± SD)
58.8 ± 22.5 g and SVL 12.2 ± 1.8 cm, respectively.

Light microscopy of blood films showed granular magenta-colored
inclusions within monocytes in 7/41 of the chameleons in this
study (Figure 1). The inclusions varied in size from approximately
3 to 12 \(\mu\)m and occasionally appeared to be composed of smaller
round-to-pleomorphic structures. The hematological data summary
is presented in Table I.

As many of the blood samples obtained were low in volume
due to the small size of the animals, the available blood was
divided between the VSMLP (\(n = 27:12F/15M\)) and the iSTAT
(\(n = 177F/10M\)) analyses. The VSARP failed to report measurements
for several analytes, including CK (10/41) and UA (1/41). All BA
(41/41) were below the built-in cut-off value of < 35 mg/dL and Ca
(3/41) and P (1/41) were above the cut-off value of > 20 mg/dL (all
females) of the VSARP. The VSMLP showed values below the report-
able range for Alb (1/41, < 1 g/dL) and BUN (19/27, < 2 mg/dL).

Individual outliers were identified for several analytes in this study
and removed from the final analysis. The biochemical and venous
samples data summary is presented in Table II. Several gender-related
significant differences were observed in some of the blood analytes
and are summarized in Table III. Agreement statistics are presented
in Table IV. The level of agreement between selected analytes mea-
sured by different tests in this study is given in Figure 2.

**Discussion**

When assessing the health of animals, clinicians and researchers
look for species-specific baseline values for blood analytes that can
be measured with relative ease using commercial blood gas and
chemistry analyzers. Species-specific hematological data is especially
important in reptiles due to the diverse environmental conditions
in different habitats that can affect the blood profiles of each spe-
cies (19). Our study provides a broad-view data set for blood gas,
biochemistry, and hematology measures in common chameleons.
Table I. Hematology determined in common chameleons.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>Range</th>
<th>RI</th>
<th>Lower limit 90% CI</th>
<th>Upper limit 90% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV (centrifuged)</td>
<td>%</td>
<td>40</td>
<td>28</td>
<td>27.5</td>
<td>6.5</td>
<td>18 to 60</td>
<td>14 to 41</td>
<td>12 to 17</td>
<td>37 to 44</td>
</tr>
<tr>
<td>TS (refractometry)</td>
<td>g/dL</td>
<td>41</td>
<td>5.6</td>
<td>5.4</td>
<td>1.3</td>
<td>3.2 to 8.8</td>
<td>2.7 to 8.2</td>
<td>2.2 to 3.3</td>
<td>7.5 to 8.9</td>
</tr>
<tr>
<td>Hct (iSTAT)</td>
<td>%</td>
<td>17</td>
<td>26</td>
<td>26</td>
<td>5</td>
<td>19 to 34</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hb (iSTAT)</td>
<td>g/dL</td>
<td>17</td>
<td>8.7</td>
<td>8.8</td>
<td>1.7</td>
<td>6.5 to 11.6</td>
<td>6.6 to 11.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>WBC*</td>
<td>×10^3/L</td>
<td>41</td>
<td>9.6</td>
<td>6.8</td>
<td>8.3</td>
<td>1.1 to 36.1</td>
<td>1.5 to 37.5</td>
<td>1.0 to 2.0</td>
<td>25.0 to 56.2</td>
</tr>
<tr>
<td>Heterophils*</td>
<td>%</td>
<td>39</td>
<td>46.2</td>
<td>42.0</td>
<td>21.4</td>
<td>12.0 to 94.0</td>
<td>12.8 to 97.0</td>
<td>0.0 to 16.2</td>
<td>81.4 to 100.0</td>
</tr>
<tr>
<td>Lymphocytes*</td>
<td>%</td>
<td>39</td>
<td>28.6</td>
<td>24.0</td>
<td>16.6</td>
<td>5.0 to 65.0</td>
<td>2.6 to 72.0</td>
<td>0.7 to 5.6</td>
<td>62.1 to 84.7</td>
</tr>
<tr>
<td>Monocytes (including azurophils)</td>
<td>%</td>
<td>39</td>
<td>14.8</td>
<td>11.0</td>
<td>12.5</td>
<td>0.0 to 50.0</td>
<td>0.0 to 37.4</td>
<td>0.0 to 0.0</td>
<td>28.9 to 46.2</td>
</tr>
<tr>
<td>Azurophils</td>
<td>%</td>
<td>39</td>
<td>5.8</td>
<td>4.0</td>
<td>5.5</td>
<td>0.0 to 22.0</td>
<td>0.0 to 17.0</td>
<td>0.0 to 0.0</td>
<td>13.1 to 19.9</td>
</tr>
<tr>
<td>Eosinophils*</td>
<td>%</td>
<td>39</td>
<td>1.9</td>
<td>0.0</td>
<td>3.6</td>
<td>0.0 to 12.0</td>
<td>0.0 to 9.2</td>
<td>0.0 to 0.0</td>
<td>5.9 to 11.7</td>
</tr>
<tr>
<td>Basophils*</td>
<td>%</td>
<td>39</td>
<td>1.8</td>
<td>1.0</td>
<td>2.7</td>
<td>0.0 to 10.0</td>
<td>0.0 to 7.4</td>
<td>0.0 to 0.0</td>
<td>5.0 to 9.2</td>
</tr>
<tr>
<td>Rubricytes</td>
<td>×10^3/L</td>
<td>35</td>
<td>51.5</td>
<td>25.0</td>
<td>44.6</td>
<td>0.0 to 175.0</td>
<td>0.0 to 126.9</td>
<td>0.0 to 0.0</td>
<td>90.2 to 158.4</td>
</tr>
<tr>
<td>Heterophils</td>
<td>×10^3/L</td>
<td>39</td>
<td>4.0</td>
<td>3.0</td>
<td>3.0</td>
<td>0.4 to 10.8</td>
<td>0.4 to 12.7</td>
<td>0.3 to 0.6</td>
<td>9.8 to 15.7</td>
</tr>
<tr>
<td>Band heterophils</td>
<td>×10^3/L</td>
<td>40</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 to 0.0</td>
<td>0.0 to 0.0</td>
<td>0.0 to 0.0</td>
<td>0.0 to 0.0</td>
</tr>
<tr>
<td>Lymphocytes*</td>
<td>×10^3/L</td>
<td>38</td>
<td>2.5</td>
<td>1.3</td>
<td>2.8</td>
<td>0.2 to 12.3</td>
<td>0.2 to 13.7</td>
<td>0.1 to 0.3</td>
<td>8.4 to 22.3</td>
</tr>
<tr>
<td>Monocytes (including azurophils)*</td>
<td>×10^3/L</td>
<td>39</td>
<td>1.9</td>
<td>0.6</td>
<td>2.6</td>
<td>0.0 to 10.8</td>
<td>0.0 to 10.2</td>
<td>0.0 to 0.0</td>
<td>5.2 to 16.0</td>
</tr>
</tbody>
</table>

*Box-Cox.

SD — standard deviation; RI — reference intervals for hematological analytes determined using a robust method with or without Box-Cox transformation; CI — confidence interval; PCV — packed cell volume; TS — total solids; Hct — hematocrit; Hb — hemoglobin; WBC — white cell count; NA — not applicable.

Although the relatively small sample size in this study (n = 41) is less than the ideal sample size (n > 120) and thus precludes the calculation of formal reference intervals (24), these results provide a useful starting point for clinicians and researchers (28) and are in line with what is often available when working with wild species (19).

Due to technical challenges in the field, primarily the low volume of the blood samples obtained in this species, total WBC counts were not done using a hemocytometer. Blood smears were used for estimating WBC counts and the differential, but were also important for evaluating cell morphology and the potential presence of blood parasites (6,7). Careful attention was placed on proper smear preparation to create a fine monolayer of cells with minimal cell lysis and disturbance of the relative cell distribution in the blood. Because cell counts are based on the relative number of cells, the estimated WBC can be influenced by the PCV and, in cases of anemic or dehydrated animals, a correction formula can be used: Total WBC × Actual PCV ÷ Normal PCV = Corrected WBC (6,7). Blood smears should ideally be prepared immediately from fresh uncoagulated samples. Heparin was used in this study as it is considered the anticoagulant of choice in reptiles in general (6,7), but its effect on the blood properties of common chameleons is yet to be determined.

The chameleons in this study were sampled from the ventral (coccygeal) tail vein, which is a venipuncture site commonly used in lizard species (4,16,29–31). As concerns about this venipuncture site include traumatic tissue injuries and transient darkening of the tail after sample collection, jugular sampling was advocated as a replacement venipuncture site (4,31). However, a comparative report did not demonstrate any major differences in measurements between samples obtained from either the tail vein or the jugular in this species (4). As more clinicians are familiar with using this venipuncture site, the tail vein was chosen for consistency to be used in this study.

This study describes several hematological analytes in common chameleons. The PCV values in this study are similar and the WBC estimate is lower than values previously reported for this species (3). The differences in the WBC between the reports can be either due to the use of a different cytological estimation technique or because some of the animals in the other study were reported to be “rescued” and potentially sick (3). In addition, reference intervals may differ in the same species depending on the season, geographical location, and captivity status (6).

Some chameleons in this study (17%) were observed to have intracytoplasmic inclusion bodies in their monocytes. This novel observation resembles Chlamydia-like inclusion bodies in the monocytes of a flap-necked chameleon (Chamaeleo dilepis) from Tanzania that also had a concurrent pox virus infection with generalized disease (32,33). These intracytoplasmic inclusion bodies were not previously reported in common chameleons and further research is required to fully identify the potential pathogen of infection. If real, this infection showed no apparent disease and no measured hematological effects when infected chameleons were compared to those that were not infected.
Table II. Blood gases and biochemistry determined in common chameleons.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Unit</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>Range</th>
<th>RI</th>
<th>Lower limit</th>
<th>Upper limit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VetScan Avian-Reptile Biochemistry Profile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase*</td>
<td>U/L</td>
<td>40</td>
<td>456</td>
<td>425</td>
<td>189</td>
<td>156 to 899</td>
<td>167 to 928</td>
<td>135 to 210</td>
<td>798 to 1076</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>U/L</td>
<td>31</td>
<td>4398</td>
<td>4320</td>
<td>1813</td>
<td>1369 to 7955</td>
<td>563 to 8128</td>
<td>0 to 1407</td>
<td>7012 to 9051</td>
</tr>
<tr>
<td>Uric acid*</td>
<td>mg/dL</td>
<td>39</td>
<td>4.6</td>
<td>3.7</td>
<td>3.1</td>
<td>1.1 to 13.4</td>
<td>1.0 to 13.8</td>
<td>0.8 to 1.3</td>
<td>10.5 to 17.6</td>
</tr>
<tr>
<td>Glucose*</td>
<td>mg/dL</td>
<td>41</td>
<td>275</td>
<td>272</td>
<td>31</td>
<td>194 to 375</td>
<td>217 to 344</td>
<td>206 to 229</td>
<td>326 to 362</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/dL</td>
<td>38</td>
<td>13.4</td>
<td>12.8</td>
<td>3.0</td>
<td>9.3 to 19.0</td>
<td>7.0 to 19.5</td>
<td>5.5 to 8.1</td>
<td>17.8 to 20.8</td>
</tr>
<tr>
<td>Phosphorus*</td>
<td>mg/dL</td>
<td>40</td>
<td>8.8</td>
<td>9.0</td>
<td>2.6</td>
<td>3.6 to 15.7</td>
<td>3.5 to 14.1</td>
<td>2.4 to 4.7</td>
<td>12.9 to 15.3</td>
</tr>
<tr>
<td>Ca:P*</td>
<td></td>
<td>37</td>
<td>1.7</td>
<td>1.5</td>
<td>0.6</td>
<td>0.6 to 3.3</td>
<td>0.7 to 3.2</td>
<td>0.6 to 0.9</td>
<td>2.7 to 3.8</td>
</tr>
<tr>
<td>Total protein</td>
<td>g/dL</td>
<td>40</td>
<td>5.1</td>
<td>5.2</td>
<td>1.0</td>
<td>2.9 to 7.2</td>
<td>3.2 to 7.2</td>
<td>2.8 to 3.7</td>
<td>6.8 to 7.7</td>
</tr>
<tr>
<td>Albumin*</td>
<td>g/dL</td>
<td>41</td>
<td>2.2</td>
<td>2.1</td>
<td>0.5</td>
<td>1.1 to 3.7</td>
<td>1.2 to 3.2</td>
<td>1.1 to 1.4</td>
<td>3.0 to 3.5</td>
</tr>
<tr>
<td>Globulin</td>
<td>g/dL</td>
<td>41</td>
<td>3.0</td>
<td>3.1</td>
<td>0.7</td>
<td>1.4 to 4.4</td>
<td>1.5 to 4.5</td>
<td>1.2 to 1.9</td>
<td>4.2 to 4.9</td>
</tr>
<tr>
<td>Potassium</td>
<td>mmol/L</td>
<td>41</td>
<td>7.3</td>
<td>7.1</td>
<td>1.2</td>
<td>4.7 to 10.0</td>
<td>4.7 to 9.8</td>
<td>4.1 to 5.2</td>
<td>9.3 to 10.3</td>
</tr>
<tr>
<td>Sodium</td>
<td>mmol/L</td>
<td>41</td>
<td>143</td>
<td>142</td>
<td>6.5</td>
<td>129 to 159</td>
<td>129 to 155</td>
<td>126 to 132</td>
<td>153 to 158</td>
</tr>
<tr>
<td>Bile acids</td>
<td>μmol/L</td>
<td>41</td>
<td>&lt; 35</td>
<td>&lt; 35</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>VetScan Mammalian Liver Profile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase*</td>
<td>U/L</td>
<td>27</td>
<td>11</td>
<td>11</td>
<td>4.7</td>
<td>2 to 23</td>
<td>1 to 21</td>
<td>0 to 4</td>
<td>17 to 23</td>
</tr>
<tr>
<td>Alanine aminotransferase*</td>
<td>U/L</td>
<td>26</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>5 to 26</td>
<td>4 to 44</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase*</td>
<td>U/L</td>
<td>26</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>0 to 24</td>
<td>0 to 17</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Bile acids</td>
<td>μmol/L</td>
<td>26</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0 to 6</td>
<td>0 to 7</td>
<td>0 to 0</td>
<td>6 to 8</td>
</tr>
<tr>
<td>Total bilirubin*</td>
<td></td>
<td>27</td>
<td>1.4</td>
<td>1.7</td>
<td>0.8</td>
<td>0.4 to 2.7</td>
<td>0.1 to 4.6</td>
<td>0.1 to 0.4</td>
<td>3.2 to 5.9</td>
</tr>
<tr>
<td>Albumin*</td>
<td>g/dL</td>
<td>25</td>
<td>1.8</td>
<td>1.8</td>
<td>0.4</td>
<td>1.2 to 2.8</td>
<td>1.0 to 2.7</td>
<td>0.0 to 1.2</td>
<td>2.4 to 2.9</td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td>mg/dL</td>
<td>27</td>
<td>NA</td>
<td>&lt; 2</td>
<td>NA</td>
<td>&lt; 2 to 2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mg/dL</td>
<td>26</td>
<td>259</td>
<td>272</td>
<td>77</td>
<td>111 to 379</td>
<td>95 to 426</td>
<td>56 to 139</td>
<td>390 to 465</td>
</tr>
<tr>
<td><strong>iSTAT CGB+ Profile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>17</td>
<td>7.17</td>
<td>7.13</td>
<td>0.19</td>
<td>6.9 to 7.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Partial pressure of carbon dioxide (PaCO₂)</td>
<td>mmHg</td>
<td>17</td>
<td>39.7</td>
<td>39.0</td>
<td>11.6</td>
<td>20.5 to 69.6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Partial pressure of oxygen (PaO₂)</td>
<td>mmHg</td>
<td>17</td>
<td>101</td>
<td>98</td>
<td>32</td>
<td>61 to 180</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Base excess</td>
<td></td>
<td>17</td>
<td>16</td>
<td>16</td>
<td>6</td>
<td>8 to 25</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Bicarbonate (HCO₃⁻)</td>
<td>mmol/L</td>
<td>17</td>
<td>23.9</td>
<td>18.1</td>
<td>13.8</td>
<td>5.6 to 44.7</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Total carbon dioxide (TCO₂)</td>
<td>mmol/L</td>
<td>17</td>
<td>17.3</td>
<td>16.0</td>
<td>9.7</td>
<td>6.0 to 38.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Saturation of oxygen (SO₂)</td>
<td>%</td>
<td>17</td>
<td>94</td>
<td>96</td>
<td>6</td>
<td>76 to 100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Sodium</td>
<td>mmol/L</td>
<td>17</td>
<td>135</td>
<td>133</td>
<td>5</td>
<td>126 to 151</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Potassium</td>
<td>mmol/L</td>
<td>17</td>
<td>5.9</td>
<td>5.5</td>
<td>0.9</td>
<td>4.3 to 7.8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ionized calcium</td>
<td>mmol/L</td>
<td>17</td>
<td>1.4</td>
<td>1.3</td>
<td>0.3</td>
<td>1.0 to 2.1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Glucose</td>
<td>mg/dL</td>
<td>16</td>
<td>281</td>
<td>277</td>
<td>31</td>
<td>246 to 369</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Reference intervals (RI) for biochemical analytes determined using a robust method with or without Box-Cox transformation.

* Box-Cox.
SD — standard deviation; CI — confidence interval; Ca:P — Calcium-to-phosphorus ratio; NA — not applicable.

A pre-determined mammalian liver profile was also used in this study as it offers several parameters not found in the VSARP. Some of these parameters are either not useful, however, or their efficacy to evaluate the reptilian liver is yet to be determined (6,7). The alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities may increase in liver disease, but may not be specific (6,7). Although usually found in very low plasma concentrations in reptiles, gamma-glutamyl transferase (GGT) can be an indicator for hepatic or renal disease (7). Cholesterol is a good marker for liver disease, such as hepatic lipidosis, but can also vary depending on the diet and can increase in the reproductive female due to vitellogenesis (6,7). As observed in this study, the blood urea nitrogen (BUN) concentrations are usually low in the uricotelic terrestrial reptiles, but can increase due to dehydration in some chelonian species (6,7).

Baseline total bilirubin concentration was measured in this study. Reptiles do not usually produce high plasma bilirubin concentrations as they lack the biliverdin reductase enzyme and biliverdin is the end product of hemoglobin metabolism (6,7). For example, total bilirubin concentration measured in Mediterranean tortoises showed near-zero results (34), but both total and direct bilirubin
were measured in desert tortoises (Gopherus agassizi) with gender and seasonal-dependent variations (35). The clinical relevance of the measured bilirubin in this study is yet to be determined as is the validity of these results, although similar findings were also reported in Negev desert tortoises (Testudo werneri) tested using the same methodology (9). It may support this theory and are also in agreement with BA reported in Negev desert tortoises tested using the same methodology (9). It is possible, however, that these assays do not measure the bile acid type(s) produced by this species. Future studies should compare different testing methodologies or test animals with known liver disease and increased plasma bile acid concentrations.

Significant gender-related differences were observed in several blood analytes in this study. For reasons unknown, measurable plasma GGT concentrations were observed only in male chameleons in this study, which was also reported in male Negev desert tortoises tested using the same methodology (9). The plasma concentrations of the AST and CK in this study were higher than those previously reported in this species (3) and are suggestive of tissue damage that released these enzymes into the blood sample (6). However, male chameleons in this study showed higher plasma CK concentrations. Reports in several chelonian species showed higher concentrations of AST and CK in males during the warm months, which is attributed to hyperactivity during the mating season. This might also be true for male common chameleons in this study, which were tested during the summer than (6) tested using the same methodology (9). Absolute values for bile acids (BA) were not recorded in any of the chameleons in this study when tested using the VSARP, which is similar to other studies in reptiles using this test (8–10,12,13,16). The VSARP measures bile acids, but has a calibrated cut-off value of 35 μmol/L. It has been suggested that most healthy reptiles would have concentrations lower than this concentration (6) and that same theory might also apply to the common chameleons sampled in this study. The low plasma concentrations of BA measured by the VSMLP may support this theory and are also in agreement with BA reported in Negev desert tortoises tested using the same methodology (9). It is also possible, however, that these assays do not measure the bile acid type(s) produced by this species. Future studies should compare different testing methodologies or test animals with known liver disease and increased plasma bile acid concentrations.

Table III. Gender-related significant differences observed in chameleons.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Unit</th>
<th>Females (n = 18) Mean/median ± SD/(IQ)</th>
<th>Males (n = 23) Mean/median ± SD/(IQ)</th>
<th>P-value (Holm adjustment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>g/dL</td>
<td>6.4 ± 1.3</td>
<td>5.0 ± 1.0</td>
<td>0.001</td>
</tr>
<tr>
<td>TP</td>
<td>g/dL</td>
<td>5.8 ± 1.2</td>
<td>4.8 ± 0.8</td>
<td>0.015</td>
</tr>
<tr>
<td>AST*</td>
<td>U/L</td>
<td>378 (164)</td>
<td>508 (208)</td>
<td>0.013</td>
</tr>
<tr>
<td>GGT*</td>
<td>U/L</td>
<td>0 (0)</td>
<td>8 (5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ca*</td>
<td>mg/dL</td>
<td>16.8 (3.3)</td>
<td>11.6 (3.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mg/dL</td>
<td>335 ± 63</td>
<td>214 ± 64</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

SD — standard deviation; IQ — interquartile; TS — total solids; TP — total proteins; AST — aspartate aminotransferase; GGT — gamma-glutamyl transferase; Ca — calcium.

* Non-parametric statistics: median and Wilcoxon tests.

Table IV. Agreement statistics between selected variables using a Passing-Bablok linear regression analysis in common chameleons.

<table>
<thead>
<tr>
<th>Pair of analytes</th>
<th>N</th>
<th>Constant bias (intercept)</th>
<th>95% CI</th>
<th>Proportional bias (slope)</th>
<th>95% CI</th>
<th>95% LOA</th>
<th>p</th>
<th>WCAEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS versus TP (VSARP)</td>
<td>41</td>
<td>0.84*</td>
<td>−0.03 to 1.67</td>
<td>0.79</td>
<td>0.64 to 0.96</td>
<td>−1.19 to 1.87</td>
<td>0.83</td>
<td>No</td>
</tr>
<tr>
<td>PCV versus Hct (iSTAT)</td>
<td>17</td>
<td>3.86*</td>
<td>−18; 13.38</td>
<td>0.71</td>
<td>0.38 to 1.5</td>
<td>−7.89; 18.01</td>
<td>0.73</td>
<td>No</td>
</tr>
<tr>
<td>Albumin (VSARP versus VSMLP)</td>
<td>25</td>
<td>−0.3*</td>
<td>−0.7; −0.3</td>
<td>1</td>
<td>1.0 to 1.2</td>
<td>0.1 to 0.6</td>
<td>0.95</td>
<td>No</td>
</tr>
<tr>
<td>Sodium (VSARP versus iSTAT)</td>
<td>17</td>
<td>61*</td>
<td>25 to 120</td>
<td>0.5*</td>
<td>0.1 to 0.8</td>
<td>−3 to 19</td>
<td>0.33</td>
<td>No</td>
</tr>
<tr>
<td>Potassium (VSARP versus iSTAT)</td>
<td>17</td>
<td>−1.6</td>
<td>−7.1 to 3.4</td>
<td>1.1</td>
<td>0.3 to 1.8</td>
<td>−0.6 to 3.8</td>
<td>0.40</td>
<td>No</td>
</tr>
<tr>
<td>Glucose (VSARP versus iSTAT)</td>
<td>16</td>
<td>17</td>
<td>−27 to 84</td>
<td>0.9</td>
<td>0.7 to 1.1</td>
<td>−25 to 31</td>
<td>0.78</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Significant bias was encountered.

CI — confidence interval; LOA — limits of agreement; p — Spearman correlation coefficient; WCAEL — within clinical allowable error limits; TS — total solids; TP — total proteins; PCV — packed cell volume; VSARP — VetScan avian/reptile profile; VSMLP — VetScan mammalian liver profile; Hct — hematocrit.

All p had a P-value of < 0.001.
which is earlier than the described mating season (mid-August to mid-September) and egg-laying (mid-September to early November) (14,39). Copulation in wild common chameleons in Israel has been reported to take place from July to September (2) and the findings of this study may also support an earlier reproductive period or pre-ovulatory activity for female common chameleons in this geographical location.

Ionized calcium was measured in chameleons in this study using the iSTAT POC analyzer and this patient-side testing methodology was advocated for this purpose in the literature (7). The ionized calcium plasma concentrations measured in chameleons in this study (1.3 ± 0.3 mmol/L, mean ± SD) are generally in agreement with other limited data described in other reptile species (6,9,16,19).

Blood gases are rarely reported in reptiles but were measured in common chameleons in this study. Recently, several studies were published using the iSTAT for determining blood gases in reptiles (16–19,21). The normal blood pH of reptiles in general is suggested to be 7.5 to 7.7 at temperatures of 23°C to 25°C and some snakes and lizards can show pH values lower than 7.4 (6). The chameleons in this study showed a mean blood pH of 7.17 (± 0.17), which can either be normal for this species or lower due to higher ambient temperatures or excitement from the venipuncture restraint (6). Studies in Galapagos marine iguanas (Amblyrhynchus cristatus) and bearded dragons (Pogona vitticeps) reported similar pH ranges using the iSTAT testing methodology (16,19). While total CO₂ measurements in reptiles are expected to range from 20 to 30 mmol/L (6), many chameleons in this study showed lower values, as was also reported in bearded dragons tested with the iSTAT methodology (16). These total CO₂ values can either be normal for common chameleons or be caused by hyperventilation from the excitement observed during the manual restraint required for the venipuncture.

Measurements of several analytes were compared when different analyses were used for their determination in this study. There was a poor level of agreement between the TS and the TP when using the VSARP , with TS being higher, likely from measuring the total solutes in the plasma (glucose, lipids, etc.). The observed disagreement between the measurements of the albumin using the VSARP and the VSMLP had a constant bias and applying a correcting factor of −0.3 can provide a good agreement between the 2 different

![Figure 2. Comparison plots for different pairs of variables measured in common chameleons: A — packed cell volume (PCV)/hematocrit (Hct) [%]; B — glucose (mg/dL); C — total solids/total protein (TS/TP) (VetScan x2) [g/dL]; D — albumin (g/dL); E — potassium (K) VetScan vs iSTAT (mmol/L); and F — sodium (Na) VetScan vs iSTAT (mmol/L). The thick dashed line represents the line of perfect agreement (y = x), the thick plain line represents the regression line (mean bias), the thin lines represent the clinically allowable error limits, and the thin dashed lines represent the 95% limits of agreement.](image)
analyses. For albumin determination, however, both the VetScan and the iSTAT analyzers use the bromocresol green (BCG) dye-binding method, which is currently considered unreliable for measuring protein in reptiles. Instead, protein electrophoresis is the recommended method for accurate measurements of plasma proteins (6,12,38,40,41). Significant disagreements were also observed between the 2 analyzers in measurements of Na and K concentrations. Good agreement was observed in blood glucose concentrations measured by the 2 analyzers, however, which was also previously described in a comparison study in black-tailed prairie dogs (Cynomys ludovicianus) (42). The disagreement observed in the higher PCV when compared to the calculated hematocrit (Hct, iSTAT) is expected and was previously described in humans and other species. It is probably due to the effect of the low plasma proteins in reptiles (<6 g/dL), which lowers the Hct results when using the iSTAT methodology (9,43). Due to these potential interferences in measurement, a spun PCV is considered more accurate than a calculated Hct (9).

As blood testing in the current study was done during the summer months and as seasonality can have a significant effect on multiple blood analytes in chameleons (31), the results might reflect what is true for this species at this time of year. Future studies should therefore test common chameleons at different seasons for comparison. The iSTAT methodology used in this study was originally designed for human blood and this may not agree with reptiles, mainly in terms of blood protein concentrations, RBC count, and auto-correction for the human body temperature (37°C) (44). The importance of doing temperature correction for several parameters (pH, PaCO₂, PaO₂) has been highlighted in reptiles and, although several differences showed when correcting for temperature in Galapagos green sea turtles (Chelonia mydas), only minor and non-clinical differences were found (18). Temperature validation was not carried out in this study as the small body size of these chameleons did not allow large volumes of blood to be collected or rectal temperatures to be tested in most animals. Although these poikilothermic chameleons were sampled during the hot summer with ambient temperatures adequate for the iSTAT blood gases calculations (25°C to 32°C), it is recommended that future studies consider temperature validation of these blood gas results.

In conclusion, data reported in this study represent an important step toward determining the normal range of physiological values against which future blood gas, biochemistry, and hematology results can be compared in common chameleons. Such assessments are important for monitoring health and diagnosing disease. The results of this study add to a growing database of knowledge about health management in chameleon species. Differences in gender and testing methodologies exist and should be considered when interpreting diagnostic data.

Acknowledgments

The authors thank Hadas Keter-Katz and Tidhar Lev-Ari from the University of Haifa, Israel and Dr. Tammy Keren-Rotem from the Israel Nature and Parks Authority for assisting with this project. This study was partially funded by an internal research grant from the Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University.

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26S proteasome and insulin-like growth factor-1 in serum of dogs suffering from malignant tumors

Ingrid Gerke, Franz-Josef Kaup, Stephan Neumann

Abstract

Studies in humans have shown that the ubiquitin-proteasome pathway and the insulin-like growth factor axis are involved in carcinogenesis, thus, components of these systems might be useful as prognostic markers and constitute potential therapeutic targets. In veterinary medicine, only a few studies exist on this topic. Here, serum concentrations of 26S proteasome (26SP) and insulin-like growth factor-1 (IGF-1) were measured by canine enzyme-linked immunosorbent assay (ELISA) in 43 dogs suffering from malignant tumors and 21 clinically normal dogs (control group). Relationships with tumor size, survival time, body condition score (BCS), and tumor entity were assessed. The median 26SP concentration in the tumor group was non-significantly higher than in the control group. However, dogs with mammary carcinomas displayed significantly increased 26SP levels compared to the control group and dogs with tumor size less than 5 cm showed significantly increased 26SP concentrations compared to dogs with larger tumors and control dogs. 26SP concentrations were not correlated to survival time or BCS. No significant difference in IGF-1 levels was found between the tumor group and the control group; however, IGF-1 concentrations displayed a larger range of values in the tumor group. Dogs with tumors greater than 5 cm showed significantly higher IGF-1 levels than dogs with smaller tumors. The IGF-1 concentrations were positively correlated to survival time, but no correlation with BCS was found. Consequently, serum 26SP concentrations seem to be increased in some dogs suffering from malignant tumors, especially in dogs with mammary carcinoma and smaller tumors. Increased serum IGF-1 concentrations could be an indication of large tumors and a poor prognosis.

Résumé

Des études chez l’human ont démontré que la voie ubiquitine-protéasome et l’axe du facteur de croissance apparenté à l’insuline sont impliqués dans la carcinogénèse, ainsi, des composantes de ces systèmes pourraient être utiles en tant que marqueurs du pronostic et constituer des cibles thérapeutiques potentielles. En médecine vétérinaire, seules quelques études existent sur ce sujet. Dans cette étude, les concentrations sériques de protéasome 26S (26SP) et du facteur de croissance 1 apparenté à l’insuline (IGF-1) ont été mesurées par réaction immunoenzymatique (ELISA) chez 43 chiens souffrant de tumeurs malignes et 21 chiens cliniquement normaux (groupe témoin). Les associations entre la taille des tumeurs, le temps de survie, le pointage de la condition corporelle (PCC) et le type de tumeurs ont été évaluées. La concentration médiane de 26SP dans le groupe avec tumeur était plus élevée que celle du groupe témoin mais de manière non-significative. Toutefois, les chiens avec des carcinomes mammaires montraient des quantités significativement augmentées de 26SP comparativement au groupe témoin et les chiens avec des tumeurs dont la taille était de moins de 5 cm avaient des concentrations de 26SP significativement augmentées comparativement aux chiens avec des tumeurs plus grosses et aux chiens du groupe témoin. Les concentrations de 26SP n’étaient pas corréllées au temps de survie ou au PCC. Aucune différence significative dans les niveaux d’IGF-1 ne fut trouvée entre le groupe avec tumeur et le groupe témoin; toutefois, les concentrations d’IGF-1 s’étendaient sur un plus large spectre dans le groupe avec tumeur. Les chiens avec des tumeurs plus grande que 5 cm avaient des concentrations d’IGF-1 significativement plus élevées que les chiens avec des tumeurs plus petites. Les concentrations d’IGF-1 étaient corrélées positivement avec le temps de survie, mais aucune corrélation avec le PCC ne fut trouvée. Conséquemment, les concentrations de 26SP semblent être augmentées chez quelques chiens souffrant de tumeurs malignes, et plus spécialement les chiens avec des carcinomes mammaires et des plus petites tumeurs. Des concentrations augmentées d’IGF-1 pourraient être une indication d’une grosse tumeur et d’un pronostic sombre.

(Inclus par Docteur Serge Messier)
Introduction

The 26S proteasome (26SP) is a large multiprotein complex, which consists of the central, cylindrical and proteolytically active 20S proteasome and 2 regulatory 19S-complexes (1). It constitutes the central part of the ubiquitin-proteasome pathway (UPP), which is the major pathway for selective protein degradation in eukaryotic cells. Thus, 26SP is involved in several cellular regulatory processes, like cell cycling, apoptosis, proliferation, differentiation, gene transcription, signal transduction, and antigen processing (2-4). During the past 3 decades, the proteasome and the UPP have been of great interest in the context of cancer research in humans. Increased circulating proteasome (c-proteasome) concentrations were found in different types of cancer in humans, including hematologic malignancy, malignant melanoma, and other solid tumors (5-10). Additionally, c-proteasome levels have been described as a prognostic factor in patients with cancer (7,9,10) and are positively correlated with the tumor burden (5,10). The origin and function of c-proteasomes are still unclear (9), but it has been shown that c-proteasomes are intact and enzymatically active (11). Furthermore, proteasome inhibitors have delivered promising results in the treatment of multiple myeloma (12-14).

The ubiquitin-proteasome pathway is also the most important proteolytic pathway in the accelerated degradation of muscle proteins in catabolic conditions (15,16). Therefore, the role of the UPP in cancer cachexia is also subject to current research in human medicine. Increased mRNA levels for components of the UPP and increased activity of muscle proteasomes were reported in animal models and in muscle tissue from patients with cancer (17).

Likewise, the insulin-like growth factor (IGF) axis and its importance in carcinogenesis (18) and cancer cachexia (19) are subjects of research in human medicine. Insulin-like growth factor-1, a 70-amino-acid polypeptide hormone (20,21), is synthesized in the liver as an anabolic hormone and also in many other organs and tissues in which this local IGF-1 has paracrine and autocrine effects. Insulin-like growth factor-1 plays a critical role in apoptosis, cell cycle control, cell proliferation, and differentiation and is highly relevant in the regulation of growth and metabolism (22). The most important functions of the IGF axis in the context of carcinogenesis are stimulation of cell proliferation, inhibition of apoptosis, and influence on cell transformation by synthesis of several regulatory proteins. Survival, clonal expansion, and metastasis of cells are influenced by components of the IGF axis. High IGF-1 concentrations have been described in the context of increased risk of cancer and, particularly, in patients with cancer (18). Queiroga et al (23) detected significantly increased IGF-1 serum levels in dogs with malignant mammary cancer and described a link between high IGF-1 serum levels and a worse prognosis. Furthermore, the IGF-1 system is downregulated in cancer cachexia, including decreased IGF-1 serum concentrations, in a rat-model with hepatoma (19). In the context of therapeutic options of cancer cachexia, intramuscular delivery of plasmid-mediated growth hormone-releasing hormone resulted in an increase of circulating IGF-1 levels and showed promising results in a study with dogs suffering from cancer cachexia (24).

In the field of veterinary medicine, only a few studies have investigated circulating IGF-1 concentrations in the context of cancer (23–25), and, to our knowledge, no studies exist on circulating proteasome concentrations in animals with cancer. Thus, the purpose of this study was to analyze 26SP proteasome and IGF-1 serum concentrations in dogs suffering from different types of cancer compared with control dogs and in relation to their tumor size, survival time, body condition score (BCS), and tumor entity. We expected increased 26SP serum concentrations in tumor patients, possibly with higher levels in large tumors and a negative correlation with the survival time. Additionally, we predicted that patients with tumor cachexia could have higher 26SP serum levels, thus, we assumed a negative correlation between 26SP serum concentrations and BCS. For IGF-1, the controversial findings in recent studies made formulation of hypotheses less straightforward. Thus, increased IGF-1 serum levels were expected in dogs with malignant tumors, potentially with higher levels in dogs with larger tumors and a negative correlation between IGF-1 serum levels and the survival time. Also, we predicted decreased IGF-1 serum levels in patients with tumor cachexia.

Materials and methods

Animals

Forty-three dogs with different malignant tumors and 21 clinically normal dogs (control group) were included in this prospective study. These dogs presented between 2013 and 2016 to the Small Animal Clinic of the University of Goettingen for diagnostic or routine health examination. All 64 dogs received a detailed clinical examination, a complete blood cell count (CBC), and serum chemistry in order to determine their health status. Additionally, the BCS (1–9) was assessed according to the WSAVA Global Nutrition Committee. Blood samples of 26SP and IGF-1 serum concentration were taken as part of a routine health examination or as part of the diagnostic workup. Diagnosis was made for solid tumors by histological examination and for malignant lymphoma by cytology after fine needle aspiration or histological examination. Tumor size and survival time were documented and survival times longer than 500 d were fixed to 500 d. All procedures were done according to the German Animal Protection Law and were carried out under the supervision of the Animal Welfare Officer, Faculty of Agriculture, University of Goettingen.

Samples

Blood samples were collected from the cephalic vein. For the measurement of 26SP and IGF-1 serum concentrations, commercial enzyme-linked immunosorbent assay (ELISA) kits were used (26SP; BlueGene Biotech, Shanghai, China; IGF-1; Cloud-Clone Corporation, Houston, Texas, USA). Following the manufacturer’s instructions, samples were collected in serum separator tubes (Sarstedt AG & Co, Nümbrecht, Germany) and were allowed to clot at room temperature for 2 h, followed by centrifugation for 15 min (26SP) and 20 min (IGF-1) at 1000 × g. Finally, serum was extracted from the tube and stored in aliquots at −80°C until analysis. Repeated freeze-thaw cycles and a storage time of more than 2 mo for IGF-1 or 6 mo for 26SP were avoided.
Table 1. Characteristics of the control group and the malignant tumor group.

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>Age (y)</td>
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<tr>
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</tr>
<tr>
<td>Height (cm)</td>
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<td>32</td>
<td>59</td>
</tr>
<tr>
<td>BCS</td>
<td>5</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td><strong>Dogs with malignant tumors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
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<td>3</td>
<td>12</td>
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<td>Weight (kg)</td>
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<td>BCS</td>
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<table>
<thead>
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<td></td>
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<tr>
<td>Mixed breeds</td>
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<td>German spaniel</td>
<td>5</td>
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<tr>
<td>Othera</td>
<td>5</td>
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<tr>
<td><strong>Dogs with malignant tumors</strong></td>
<td></td>
</tr>
<tr>
<td>Mixed breed</td>
<td>8</td>
</tr>
<tr>
<td>Rhodesian ridgeback</td>
<td>4</td>
</tr>
<tr>
<td>German shepherd</td>
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<tr>
<td>Labrador retriever</td>
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<tr>
<td>Wire-haired dachshund</td>
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<tr>
<td>Boxer</td>
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<tr>
<td>Golden retriever</td>
<td>2</td>
</tr>
<tr>
<td>Jack Russell terrier</td>
<td>2</td>
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<tr>
<td>Otherb</td>
<td>16</td>
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</table>

<table>
<thead>
<tr>
<th>Gender and status of castration</th>
<th>Intact</th>
<th>Neutered</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
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<td>Male</td>
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<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td><strong>Dogs with malignant tumors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>7</td>
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<tr>
<td>Male</td>
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<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>12</td>
<td>43</td>
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<table>
<thead>
<tr>
<th>Tumor size</th>
<th>&lt; 5 cm</th>
<th>&gt; 5 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>53.57%</td>
<td>46.43%</td>
</tr>
</tbody>
</table>

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**26S proteasome assay**

Serum 26S proteasome concentrations were measured using a commercially available canine 26S proteasome quantitative ELISA kit (BlueGene Biotech, Shanghai, China) according to the manufacturer’s instructions. All standards and samples were applied in triplicate on the ELISA plate. The optical density (OD) of each well was analyzed at a wavelength of 450 nm using a microplate reader (TECAN microplate reader; TECAN Austria GmbH, Groeding, Austria). The serum 26S levels (ng/mL) were determined by comparing the OD of the samples to the standard curve, using computer software (CurveExpert 1.4; CurveExpert, Daniel G. Hyams, Hixson, Tennessee, USA). According to the manufacturer’s instructions, the minimum detectable concentration of 26SP in this ELISA is 0.1 ng/mL. The manufacturer indicates the mean intra- and inter-assay coefficients of variation with < 10%.

**Insulin-like growth factor-1 assay**

A canine ELISA kit for IGF-1 (Cloud-Clone Corporation) was used for measuring serum IGF-1 levels. All samples and standards
were measured in triplicate. As for 26SP, the OD of each well was analyzed by a microplate reader (TECAN microplate reader; TECAN Austria GmbH) at a wavelength of 450 nm and computer software (CurveExpert 1.4; CurveExpert) was used to generate the standard curves and calculate the IGF-1 concentrations (ng/mL) of the samples. The minimum detectable concentration of IGF-1 in this ELISA kit is typically less than 2.42 ng/mL. The manufacturer indicates the mean intra- and inter-assay coefficients of variation with 10% and 12%, respectively.

**Histology**

Histology was done on routinely embedded paraffin sections after hematoxylin and eosin (H&E) staining. Diagnosis was done by a Board-certified pathologist (DECVP).

**Statistical analysis**

Statistical analysis was done using computer software [STATISTICA, Version 12.7; StatSoft (Europe) GmbH, Hamburg, Germany]. Medians and range or maximum and minimum of values were calculated. The statistical distributions of the variables were evaluated graphically by inspections of histograms and quantile-quantile plots. Spearman’s rank correlation was used to test relationships between 26SP or IGF-1 and numeric parameters. The non-parametric Mann-Whitney U-test (MWUT) was used for comparison of 2 groups. Kruskal-Wallis analysis of variance (KWANOVA) was used for comparing more than 2 groups, in connection with MWUT as post-hoc test for pairwise comparisons. The Levene test for homogeneity of variance was used for comparing the variances of IGF-1 concentrations between the malignant tumor and control group. A P-value < 0.05 was considered statistically significant.

**Results**

**Control group**

The control group was composed of 21 dogs. Table I shows the distribution of age, weight, shoulder height, BCS, and gender in this group, as well as status of castration and the represented breeds.

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**Table II. 26SP and insulin-like growth factor-1 (IGF-1) levels according to survival time.**

<table>
<thead>
<tr>
<th>Survival time (d)</th>
<th>n</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>26SP (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 7</td>
<td>14</td>
<td>1.2</td>
<td>0.1</td>
<td>6.0</td>
</tr>
<tr>
<td>8 to 90</td>
<td>7</td>
<td>2.65</td>
<td>0.1</td>
<td>11.8</td>
</tr>
<tr>
<td>91 to 365</td>
<td>3</td>
<td>1.7</td>
<td>0.9</td>
<td>5.5</td>
</tr>
<tr>
<td>&gt; 365</td>
<td>9</td>
<td>3.6</td>
<td>0.1</td>
<td>9.9</td>
</tr>
<tr>
<td>IGF-1 (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 7</td>
<td>13</td>
<td>471.44</td>
<td>58.92</td>
<td>1200</td>
</tr>
<tr>
<td>8 to 90</td>
<td>2</td>
<td>199.61</td>
<td>182.34</td>
<td>216.87</td>
</tr>
<tr>
<td>91 to 365</td>
<td>3</td>
<td>258.36</td>
<td>43.20</td>
<td>289.15</td>
</tr>
<tr>
<td>&gt; 365</td>
<td>9</td>
<td>134.88</td>
<td>34.44</td>
<td>556.42</td>
</tr>
</tbody>
</table>

**Table III. 26SP levels (ng/mL) according to tumor entity.**

<table>
<thead>
<tr>
<th>Tumor entity</th>
<th>n</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control dogs</td>
<td>21</td>
<td>0.8</td>
<td>0.2</td>
<td>4.6</td>
</tr>
<tr>
<td>All malignant tumors</td>
<td>43</td>
<td>2.1</td>
<td>0.1</td>
<td>11.8</td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>11</td>
<td>3.7</td>
<td>0.1</td>
<td>9.9</td>
</tr>
<tr>
<td>Splenic sarcoma (6 hemangiosarcoma, 1</td>
<td>7</td>
<td>0.9</td>
<td>0.2</td>
<td>5.5</td>
</tr>
<tr>
<td>spindle cell sarcoma)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>7</td>
<td>1.1</td>
<td>0.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Lymphoma under chemotherapy</td>
<td>2</td>
<td>7.4</td>
<td>3.0</td>
<td>11.8</td>
</tr>
<tr>
<td>Other malignant tumors</td>
<td>16</td>
<td>2.2</td>
<td>0.1</td>
<td>9.7</td>
</tr>
<tr>
<td>Mastocytoma (low grade)</td>
<td>3</td>
<td>4.4</td>
<td>3.6</td>
<td>6.0</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>2</td>
<td>0.3</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>2</td>
<td>3.2</td>
<td>1.3</td>
<td>5.0</td>
</tr>
<tr>
<td>Enteral spindle cell sarcoma</td>
<td>1</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anal sac adenocarcinoma</td>
<td>1</td>
<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant gastrointestinal stromal</td>
<td>1</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal adenocarcinoma</td>
<td>1</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutaneous hemangiopericytoma</td>
<td>1</td>
<td>0.4</td>
<td></td>
<td></td>
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<tr>
<td>Thyroid gland carcinoma</td>
<td>1</td>
<td>9.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>1</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastasized hepatic sarcoma</td>
<td>1</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic hemangiosarcoma</td>
<td>1</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Neither IGF-1 nor 26SP serum concentrations were normally distributed in the control group. The 26SP concentrations ranged from 0.2 to 4.6 ng/mL with a median of 0.8 ng/mL and IGF-1 levels had a median of 265.5 ng/mL with a range from 104.4 to 641.88 ng/mL.

**Dogs with malignant tumors**

The group of dogs with malignant tumors consisted of 11 cases of mammary carcinomas, 7 splenic sarcomas (6 hemangiosarcomas and 1 spindle cell sarcoma), 9 lymphomas (2 under chemotherapy), and 16 other malignant tumors. Table I shows the distribution of age, weight, shoulder height, BCS, gender, status of castration, the represented breeds, and the tumor size (grouped into <5 cm and >5 cm) in the group of dogs with malignant tumors.

**26S proteasome**

In the group of dogs with malignant tumors, the serum 26SP concentrations did not show a normal distribution. The median was 2.1 ng/mL with a range from 0.1 to 11.8 ng/mL. Thus, the median of the 26SP concentrations was higher in dogs with malignant tumors compared with the group of control dogs (median: 0.8 ng/mL), with extremely high values occurring only in the malignant tumor group (Figure 1). However, the difference was not statistically significant ($U = 320.5, I = 0.0607$).

When dividing the dogs with malignant tumors into 2 groups based on tumor size (<5 cm and >5 cm) and comparing these groups to each other and to the control dogs, significant differences in 26SP concentrations were found ($H = 12.9, df = 2, P = 0.0016$). Pairwise comparisons showed significantly higher 26SP levels in dogs with tumors <5 cm than in dogs with tumors >5 cm ($P = 0.0099$) and control dogs ($P = 0.0027$; Figure 2).

In the group of dogs with malignant tumors, no correlation between 26SP and the BCS (Spearman $\rho = 0.1442, P = 0.4015$) or the time of survival (Spearman $\rho = 0.2596, P = 0.1659$) could be found. Table II gives an overview of 26SP levels broken down by survival time (0 to 7 d, 8 to 90 d, 91 to 365 d, and >365 d).

When examining 26SP concentrations depending on the different tumor entities (Table III), significantly increased concentrations of 26SP in dogs with mammary carcinomas compared to the control group were found ($U = 62, P = 0.0339$). For the other tumor entities, there were no indications of differences in concentrations of 26SP compared to the control group, or the group size was too small for statistical analysis. For example, the 2 dogs with lymphoma under chemotherapy showed relatively high 26SP concentrations, one of them showed a 26SP concentration of 11.8 ng/mL. Furthermore, a very high 26SP concentration was detected in a dog suffering from a thyroid gland carcinoma. High 26SP concentrations, just slightly above or in the upper range of the values of control dogs, were observed in all 3 dogs with low grade mastocytoma, in one dog with malignant melanoma, one dog with anal sac adenocarcinoma, and one dog with gastric carcinoma. Additionally, similarly high 26SP concentrations were also found in some of the dogs with splenic sarcoma and lymphoma.

**Insulin-like growth factor-1**

In the group of dogs with malignant tumors, IGF-1 concentrations were not normally distributed. For 3 dogs, no measurements of IGF-1 were available, and in 2 dogs the IGF-1 concentration in the diluted sample was higher than the highest standard and, thus, higher than 1200 ng/mL. For the purpose of analysis, these 2 values were set to 1200 ng/mL. This resulted in a median IGF-1 concentration of 251.09 ng/mL and a range from 24.36 to 1200 ng/mL. No significant difference in IGF-1 concentrations between the group of dogs with malignant tumors and the control group was found ($U = 410, P = 0.8863$). However, IGF-1 concentrations showed significantly greater variance in the group of dogs with malignant tumors ($F = 9.99, P = 0.0025$; Figure 3).

Comparing the IGF-1 concentrations of dogs with malignant tumors <5 cm to those of dogs with tumors >5 cm and control dogs indicated significant differences between the groups ($H = 7.54, df = 2, P = 0.0230$). Pairwise comparisons showed significantly higher...
IGF-1 levels in dogs with tumors > 5 cm than in dogs with tumors < 5 cm diameter \((P = 0.0187)\). However, no difference to control dogs could be found \((P_{\text{control} - \text{tumor} \leq 5 \text{ cm}} = 0.2923, P_{\text{control} - \text{tumor} < 5 \text{ cm}} = 0.6259);\) Figure 4).

Insulin-like growth factor-1 concentrations were not correlated with the BCS (Spearman \(r = -0.1168, P = 0.5173\)), but showed a negative correlation to the time of survival (Spearman \(r = -0.4617, P = 0.0153\)). Insulin-like growth factor-1 levels greater than 600 ng/mL were only found in the group of dogs which died or were euthanized within 7 d after diagnosis, but also many lower IGF-1 levels were detected in this group (Table II).

No indications of increased IGF-1 serum concentrations were found for mammary carcinomas, splenic sarcomas, or lymphomas relative to the control group (Table IV). However, a very large range was detected in the lymphoma group (34.44 ng/mL to 1200 ng/mL). The 2 dogs with lymphoma under chemotherapy showed relatively low IGF-1 levels; however, these were comparable to the lowest values in the group of dogs with lymphoma who did not receive treatment at the time of blood sampling. Additionally, we found decreased IGF-1 serum concentrations in the 3 dogs with low grade mastocytoma, but the group size was too small for statistical analysis. Furthermore, very high IGF-1 concentrations were detected in each of the single dogs with renal adenocarcinoma (816.44 ng/mL), thyroid gland carcinoma (> 1200 ng/mL), and gastric carcinoma (1170 ng/mL). The remaining dogs showed IGF-1 concentrations in the same range as the control dogs.

### Discussion

In this study, we measured 26S proteasome and IGF-1 serum concentrations in dogs suffering from different types of cancer and in control dogs using canine ELISA and analyzed them in relation to tumor size, time of survival, BCS, and tumor entity.

#### 26S-proteasome

Even though the median 26SP concentrations were higher in the group of dogs with malignant tumors compared with control dogs, and the highest 26SP values in tumor patients were more than twice as high as those of control dogs, this difference was not significant. However, when only the dogs with mammary carcinomas were considered, significantly increased 26SP concentrations were detected compared to control dogs. In humans, elevated circulating proteasome concentrations have been described in many different kinds of cancer (5–8,10), including breast carcinoma (8). Therefore, if serum 26SP levels are increased in dogs or not depends on the tumor entity, or increased 26SP levels might not only be an expression of the neoplastic disease, but might be due to another cause. Further studies with a greater number of patients in the different tumor entity subgroups are necessary for further clarification.

Moreover, the very high 26SP concentrations in the 2 dogs suffering from lymphoma under chemotherapy were conspicuous. This observation complies with the report of higher c-proteasome concentrations after chemotherapy in humans with ovarian cancer. Heubner et al (10) assumed non-selective cytotoxicity of chemotherapy as a possible reason and, thus, the healthy tissue as another potential origin of c-proteasome.

When considering tumor size, we found significantly increased 26SP levels in dogs with malignant tumors < 5 cm compared with control dogs and dogs with malignant tumors > 5 cm. Thus, 26SP serum concentrations seem to be especially increased in dogs with smaller tumors. This is in contrast to the described positive correlation between c-proteasome levels and the tumor burden in...
Insulin-like growth factor-1

Insulin-like growth factor-1 concentrations were not significantly different in the group of dogs suffering from malignant tumors compared to the control group. However, it was noticeable that the IGF-1 concentrations ranged significantly more in both directions in the malignant tumor group than in the control group. Thus, the group of dogs with malignant tumors consisted of dogs with decreased, unchanged, and increased IGF-1 levels. One possible reason could be that different kinds of tumors impact IGF-1 serum concentrations differently. However, we did not find any significant differences between the different tumor entities, and a large range of IGF-1 values was also observed within the different tumor entity groups. Here, the very small numbers of patients in the different tumor entity groups needs to be considered. For example, the 3 dogs with low grade mastocytomas showed relatively low IGF-1 values. Additionally, the low IGF-1 concentrations in the 2 dogs with lymphoma under chemotherapy were conspicuous; however, equal IGF-1 concentrations were also found in the lymphoma patients without chemotherapy. Furthermore, extremely high IGF-1 levels were detected in this group, too. Extremely high IGF-1 concentrations were also found in each of the single dogs with renal adenocarcinoma, thyroid gland carcinoma, and gastric carcinoma. The remaining dogs showed IGF-1 concentrations in the same range as the control dogs. Why IGF-1 concentrations showed such a large variation in malignant tumor patients and especially in the lymphoma group, cannot be explained based on our dataset and requires further investigation.

Controversial statements can be found in the literature regarding circulating IGF-1 concentrations in cancer patients — also within the same types of cancer. Queiroga et al (23) detected significantly increased IGF-1 serum concentrations in dogs with malignant mammary cancer. Several studies in humans have found increased circulating IGF-1 concentrations, for example, in patients with ovarian cancer (28), endometrial cancer (29), pancreatic cancer (30), breast cancer (31), lung cancer (32), and malignant melanoma (33). However, there are also many studies that reported unchanged IGF-1 concentrations, for example, in patients with pancreatic cancer (34), breast cancer (35), and endometrial cancer (36). Some studies also detected decreased IGF-1 levels, for example, in patients with endometrial cancer (37) and ovarian cancer (38), as reviewed in Kasprzak et al (18).

It cannot be excluded that other factors, such as body size and age, influenced the IGF-1 levels in our study. Generally, small dog breeds have lower IGF-1 levels than large dog breeds (39), and in humans IGF-1 shows an age dependency with higher levels during growth and slightly decreasing levels in geriatric patients (40). However, we did not detect a correlation between IGF-1 concentrations and shoulder height or the body weight in the malignant tumor group (data not shown), which consisted only of fully grown dogs. Moreover, IGF-1 levels were positively correlated with the age of the dogs (data not shown). Thus, it is unlikely that both of these factors have influenced the results herein.

Insulin-like growth factor-1 concentrations were significantly higher in dogs with tumors > 5 cm than in dogs with tumors < 5 cm. However, no significant difference to control dogs was found, although the highest IGF-1 concentrations were found only in dogs with tumors greater than 5 cm. Altogether this gives an indication of a connection between IGF-1 serum concentrations and tumor size, which is also described in human medicine (32).

A negative correlation between IGF-1 concentrations and the time of survival was detected. The IGF-1 levels > 600 ng/mL were found only in the group of dogs that died or were euthanized within 7 d after diagnosis. Thus, increased IGF-1 concentrations seem to be an indication of a poor prognosis. Many dogs which died or were euthanized within 7 d after diagnosis also showed normal IGF-1 levels. One explanation for this broad range of values in this group could be the decision of the owners to euthanize, which may be driven by factors other than prognosis (e.g., cost of therapy). Queiroga et al (23) demonstrated a link between high IGF-1 levels and a poor prognosis in dogs with mammary cancer, and a connection between high circulating IGF-1 concentrations and a poor prognosis has also been observed in humans with several types of cancer (32,33).

In this study IGF-1 concentrations were not correlated with the BCS. Thus, dogs with cancer cachexia did not show decreased IGF-1 levels. In contrast, Costelli et al (19) detected decreased IGF-1 serum levels and decreased IGF-1 production in the liver of cachectic humans (5,10). Indeed, it is possible that the significantly increased 26SP concentrations in dogs with malignant mammary carcinomas have influenced this result, because most of the mammary carcinomas were smaller than 5 cm. However, there may be additional reasons for the difference between both tumor sizes. One hypothesis explaining the increased 26SP levels in connection with smaller tumors could be that more mechanisms are active to prevent the progression of tumor growth in smaller tumors, as 26SP degrades damaged and misfolded proteins and is involved in cell cycle control and apoptosis (2–4). However, this is in contrast to the observation that proteasome-inhibitors slowed tumor progression by induction of cell-cycle arrest and apoptosis (12,26). Further studies are needed to clarify the finding of increased 26SP levels in dogs with small tumors and the underlying mechanisms.

In the malignant tumor group, there was no correlation between 26SP and survival time, thus, we did not find any indication for the usefulness of serum proteasome concentration as a prognostic factor, as it has been described for humans (7,9,10). The ubiquitin-proteasome system is presumed to play an important role in the accelerated degradation of muscle proteins in catabolic metabolic states, including cancer cachexia (15–17) and increased c-proteasome concentrations were interpreted as a marker of muscle degradation in sepsis and trauma patients (27). Thus, we assumed higher 26SP concentrations in dogs with decreased BCS, but we could not detect a correlation between 26SP and the BCS in this study.

Overall, it should be taken into account that in the studies on humans, 20SP concentrations instead of 26SP concentrations were measured, which were much higher than the concentrations we detected for 26SP, both in patients who are clinically normal and in patients suffering from cancer. Whether this is due to species-specific differences or due to the different serum concentrations of 20SP and 26SP should be clarified in further studies. 26SP consists of the 20S proteasome and 2 regulatory 19S-complexes (1) and it is possible that in the serum mainly 20SP exists. To our knowledge 26SP and 20SP should be clarified in further studies. 26SP consists of the 20S proteasome and 2 regulatory 19S-complexes (1) and it is possible that in the serum mainly 20SP exists. To our knowledge the ubiquitin-proteasome system is presumed to play an important role in the accelerated degradation of muscle proteins in catabolic metabolic states, including cancer cachexia (15–17) and increased c-proteasome concentrations were interpreted as a marker of muscle degradation in sepsis and trauma patients (27). Thus, we assumed higher 26SP levels in dogs with small tumors and the underlying mechanisms.

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rats with hepatoma and concluded that the IGF-1 system is downregulated in cancer cachexia. An alternative explanation, however, could be reduced synthesis due to impaired liver function. For example, Neumann et al (25) found decreased IGF-1 serum levels in dogs suffering from different kinds of liver diseases, including cancer and hepatitis. Maybe this is the underlying reason why we could not replicate the findings of Costelli et al (19) with regard to cancer cachexia in our study, as the dogs were suffering from different kinds of tumors and not only hepatic neoplasias.

This study indicates increased 26S proteasome concentrations in dogs suffering from several types of malignant tumors; by isolating mammary carcinomas the difference to the control group was significant. Surprisingly, especially dogs with smaller tumors showed elevated 26S proteasome levels. This might be explained by the significantly increased 26S proteasome concentrations in dogs with mammary carcinoma, because most of the mammary carcinomas were < 5 cm, and potentially by an intensification of mechanisms that prevent the progression of tumor growth. A relationship between 26S proteasome concentrations and survival time or BCS could not be detected in this study.

The IGF-1 serum concentrations were not significantly increased or decreased in the tumor group compared with the control group, but showed a significantly larger range. However, significantly increased IGF-1 serum concentrations were found in dogs with tumors > 5 cm than in dogs with tumors < 5 cm. Thus, IGF-1 serum concentrations seem to increase with tumor size. The IGF-1 levels were negatively correlated with the survival time, indicating a relationship between increased IGF-1 levels and a poor prognosis. No correlation was found between IGF-1 and the BCS. Consequently, cancer cachexia does not seem to be accompanied by decreased IGF-1 serum concentrations in dogs in all cases.

As these conclusions are based on relatively few patients, further studies on proteasome and IGF-1 in connection to cancer based on larger group sizes and more homogeneous groups are needed in veterinary research.

References


Efficacy and pharmacokinetics of bupivacaine with epinephrine or dexmedetomidine after intraperitoneal administration in cats undergoing ovariohysterectomy

Javier Benito, Beatriz Monteiro, Francis Beaudry, Paulo Steagall

Abstract

The aim of this study was to determine the efficacy and pharmacokinetics of bupivacaine in combination with epinephrine or dexmedetomidine after intraperitoneal administration in cats undergoing ovariohysterectomy. Sixteen healthy adult cats (3.3 ± 0.6 kg) were included in a prospective, randomized, masked clinical trial after obtaining owners’ consent. Anesthetic protocol included buprenorphine-propofol-isoflurane. Meloxicam [0.2 mg/kg body weight (BW)] was administered subcutaneously before surgery. Cats were randomly divided into 2 groups to receive 1 of 2 treatments. Intraperitoneal bupivacaine 0.25% (2 mg/kg BW) was administered with epinephrine (BE group; 2 μg/kg BW) or dexmedetomidine (BD group; 1 μg/kg BW) before ovariohysterectomy (n = 8/group). A catheter was placed in the jugular vein for blood sampling. Blood samples were collected for up to 8 h after bupivacaine was administered. Plasma concentrations and pharmacokinetics of bupivacaine were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS) and non-compartmental model, respectively. Pain was evaluated using the UNESP-Botucatu multidimensional composite pain scale (MCPS), the Glasgow composite feline pain scale (GPS), and a dynamic visual analog scale up to 8 h after extubation. Rescue analgesia was provided with buprenorphine if MCPS was ≥6. Repeated measures linear models were used for analysis of pain and sedation scores (P < 0.05). Maximum bupivacaine plasma concentrations (Cmax) for BE and BD were 1155 ± 168 ng/mL and 1678 ± 364 ng/mL (P = 0.29) at 67 ± 13 min (Tmax) and 123 ± 59 min (P = 0.17), respectively. Pharmacokinetic parameters and pain scores were not different between treatments (P > 0.05). One cat in the BE group received rescue analgesia (P = 0.30). Intraperitoneal bupivacaine with epinephrine or dexmedetomidine produced concentrations below toxic levels and similar analgesic effects. It is therefore safe to administer these drug combinations in cats undergoing ovariohysterectomy.

Résumé

L’objectif de cette étude était de déterminer la pharmacocinétique de la bupivaïcaine avec de l’épinéphrine ou de la dexmedetomidine après son administration intrapéritonéale chez des chats subissant une ovariohystérectomie. Seize chats adultes en bonne santé (3,3 ± 0.6 kg) ont été inclus dans un essai prospectif, randomisé et «à l’aveugle». Le protocole anesthésique comprenait la buprénorphine-propofol-isoflurane. Méloxicam (0,2 mg/kg) a été administré par voie sous-cutanée avant la chirurgie. Un cathéter a été placé dans la veine jugulaire pour l’échantillonnage du sang. La bupivaïcaine 0,25 % a été administrée intrapéritonéale (2 mg/kg) avec de l’épinéphrine (BE, 2 μg/kg) ou de la dexmedetomidine (BD, 1 μg/kg) avant l’ovariohystérectomie (n = 8/groupe). Des échantillons de sang ont été prélevés jusqu’à 8 heures après l’administration de bupivaïcaine. Les concentrations plasmatiques et les données pharmacocinétiques de la bupivaïcaine ont été déterminées par l’aide de la chromatographie liquide-spectrométrie de masse (LC-MS) et la représentation graphique avec un modèle non-compartmental. La douleur a été évaluée à l’aide de l’échelle composite multidimensionnelle de la douleur (MCPS), de l’échelle composite de Glasgow de la douleur féline (GPS) et d’une échelle visuelle analogique dynamique jusqu’à 8 heures après l’extubation. L’analgesie de secours a été fournie avec la buprénorphine si MCPS ≥ 6. Les modèles linéaires de mesures répétées ont été utilisés pour l’analyse des scores de douleur et de sédation (P < 0.05). Les concentrations plasmatiques maximales de bupivaïcaine (Cmax) pour BE et BD étaient de 1155 ± 168 ng/mL et 1678 ± 364 ng/mL (P = 0.29) à 67 ± 13 minutes (Tmax) et 123 ± 59 minutes (P = 0.17), respectivement. Les paramètres pharmacocinétiques et les scores de douleur n’étaient pas différents entre les traitements (P > 0.05). Un chat de BE a reçu analgésie de secours (P = 0.30). La bupivaïcaine avec de l’épinéphrine ou de la dexmedetomidine intra-péritonéale a produit des concentrations inférieures aux niveaux toxiques et des effets analgésiques similaires. Il est donc sécuritaire d’administrer ces combinaisons de médicaments chez les chats subissant une ovariohystérectomie.

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Received August 2, 2017. Accepted September, 2017.
Introduction

Intraperitoneal (IP) administration of local anesthetics reduces early postoperative analgesic requirements and pain scores and increases time to first intervention analgesia after abdominal surgery in humans (1,2). For these reasons, the technique has been recommended as part of a multimodal approach after laparoscopic surgery (3,4). In veterinary medicine, the technique has been shown to be a simple, safe, and cost-effective method for reducing pain after ovariohysterectomy in dogs (5–8) and cats (9,10) without adverse effects (6,8,9).

In human medicine, bupivacaine, a long-acting local anesthetic, has been administered intraperitoneally in combination with adjuvant drugs, such as agonists of α2-adrenoreceptors, e.g., dexmedetomidine and epinephrine (11,12). These adjuvant drugs produce local vasoconstriction, which delays systemic absorption and improves the safety and efficacy of the IP local anesthetic (3). The same approach could benefit feline pain management since bupivacaine, dexmedetomidine, and epinephrine are non-controlled drugs that are available worldwide. To the authors’ knowledge, the pharmacokinetics of IP bupivacaine in combination with dexmedetomidine or epinephrine has not yet been determined in cats.

The aims of this study were to determine plasma concentrations and deriving pharmacokinetics from concentration-time data plotting and to evaluate the postoperative analgesic efficacy of bupivacaine in combination with epinephrine or dexmedetomidine after IP administration in cats undergoing ovariohysterectomy. The authors hypothesized that plasma concentrations of bupivacaine would be detected in combination with dexmedetomidine or epinephrine after IP administration, adverse effects would not be observed, and these treatments would provide similar analgesic effects postoperatively. Results of this study were compared with a previous pharmacokinetic study in which bupivacaine alone was administered intraperitoneally in cats undergoing ovariohysterectomy using similar experimental conditions in our laboratory (9).

Materials and methods

The study protocol was approved by the local animal care committee (protocol number 16-Rech-1833) and conducted according to Canadian Council on Animal Care guidelines.

Experimental design and treatment groups

This study was a prospective, randomized, masked clinical trial. Cats were randomly assigned using www.randomization.org (accessed June 28, 2016) to receive 1 of the following 2 treatments by the IP route of administration (n = 8/group): the bupivacaine-epinephrine group (BE) received bupivacaine at 2 mg/kg body weight (BW) (Sensorcaine, bupivacaine HCl 0.5% USP; AstraZeneca, Mississauga, Ontario) and epinephrine at 2 μg/kgBW (Epiclor, Rafter 8; Calgary, Alberta). The bupivacaine-dexmedetomidine (BD) group received bupivacaine at 2 mg/kg BW and dexmedetomidine at 1 μg/kgBW (Dexdomitor; Zoetics, Kirkland, Quebec). In both the BE and BD groups, a solution of bupivacaine 0.5% with epinephrine (1 μg/1 mg of bupivacaine, corresponding to the dose 2 μg/kg BW) or dexmedetomidine (1 μg/kg BW), respectively, was diluted with an equal volume of isotonic sterile saline (0.9% sodium chloride USP; Hospira, Montreal, Quebec), which resulted in a final concentration of 0.25% of bupivacaine.

Study animals

Sixteen adult, mixed-breed, female cats (3.3 ± 0.6 kg) from a local animal shelter were admitted to the veterinary teaching hospital (Centre hospitalier universitaire vétérinaire (CHUV)) of the Faculty of Veterinary Medicine, Université de Montréal for elective ovariohysterectomy, after obtaining the shelter’s written consent. Cats were included in this study if they were considered healthy based on a complete physical examination and normal values for hematocrit and total protein. Exclusion criteria included body weight < 2 kg, cardiac arrhythmias, pregnancy, lactation, body condition score of > 7 or < 3 on a scale from 1 to 9, anemia (hematocrit < 25%), hypoproteinemia (total protein < 59 g/dL), and clinical signs of disease, such as upper tract respiratory infection. Study animals were housed individually in adjacent cages in the cat ward at CHUV.

Experimental procedure

Food but not water was withheld up to 8 h before general anesthesia. Approximately 20 min before induction, a 22-G catheter was inserted aseptically into a cephalic vein. Cats did not receive any premedication and anesthesia was induced using propofol intravenously (IV) (Diprivan 1%; AstraZeneca). Lidocaine 2% (0.05 mL) (Xylocaine; AstraZeneca) was instilled over the arytenoid cartilages and the cats were intubated with an appropriately sized, cuffed endotracheal tube. Anesthesia was maintained with isoflurane (Isoflurane USP; Pharmaceuticals Partners of Canada, Richmond Hill, Ontario) administered in oxygen using a non-rebreathing circuit. Cats were then positioned in dorsal recumbency on a circulating warm water blanket and monitoring [electrocardiogram (ECG), capnography, and pulse oximetry] was recorded every 5 min using a multiparametric monitor (Lifewindow 6000V veterinary multiparameter monitor; Digicare Animal Health, Boynton Beach, Florida, USA). Blood pressure was monitored using a Doppler ultrasound blood flow detector (Doppler Model 811-B; Park Electronics, Aloha, Oregon, USA). A balanced crystalloid solution was administered intravenously at a rate of 10 mL/kg BW per hour throughout anesthesia and surgery. Before surgery began, a 20-G, 1.16-in catheter was aseptically inserted into a jugular vein for blood sampling, fixed with suture, and protected with a bandage. Buprenorphine (Vetergesic; Champion Alstoe Animal Health, York, England) (0.02 mg/kg BW, IV) and meloxicam (Metacam 0.5%; Boehringer Ingelheim, Burlington, Ontario) (0.2 mg/kg BW, subcutaneously (SC)) were administered for analgesia after induction of anesthesia and before surgery.

Ovariohysterectomy was carried out by the same veterinarian (BPM) using a ventral midline incision as reported in a previous study (10). The individual withdrew the test drug (BE or BD) solution in a sterile manner using a 3-mL syringe attached to a 22-G, 1.16-in catheter and divided equally into 3 parts to be administered intraperitoneally. Specifically, each part of the solution was instilled (“splashed”) over the right and left ovarian pedicles and at the caudal aspect of the uterine body immediately after the first venous blood sample had been collected (time point 0, baseline). The ovariohysterectomy was done approximately 2 min later. Duration of surgery (time elapsed from the first incision until placement of the last suture), anesthesia
Table I. Body condition score (BCS), body weight, hematocrit, and total protein (before and after surgery), duration of anesthesia and surgery, and time to extubation in cats undergoing ovariohysterectomy after intraperitoneal administration of bupivacaine-epinephrine (BE) or bupivacaine-dexmedetomidine (BD).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Population</th>
<th>Group BE</th>
<th>Group BD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCS (1 to 9)</td>
<td>n = 16</td>
<td>5 ± 0</td>
<td>5 ± 0</td>
<td>0.57</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
<td>3.3 ± 0.6</td>
<td>3.2 ± 0.6</td>
<td>0.91</td>
</tr>
<tr>
<td>Hematocrit before (%)</td>
<td></td>
<td>37 ± 4</td>
<td>36 ± 5</td>
<td>0.70</td>
</tr>
<tr>
<td>Normal range (28 to 47)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit after (%)</td>
<td></td>
<td>31 ± 4</td>
<td>29 ± 4</td>
<td>0.45</td>
</tr>
<tr>
<td>Normal range (28 to 47)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein before (g/dL)</td>
<td></td>
<td>73 ± 8</td>
<td>72 ± 4</td>
<td>0.79</td>
</tr>
<tr>
<td>Normal range (59 to 81)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein after (g/dL)</td>
<td></td>
<td>62 ± 5</td>
<td>61 ± 5</td>
<td>0.40</td>
</tr>
<tr>
<td>Normal range (59 to 81)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of anesthesia (min)</td>
<td></td>
<td>58 ± 15</td>
<td>60 ± 21</td>
<td>0.81</td>
</tr>
<tr>
<td>Duration of surgery (min)</td>
<td></td>
<td>19 ± 2</td>
<td>19 ± 3</td>
<td>0.62</td>
</tr>
<tr>
<td>Time to extubation (min)</td>
<td></td>
<td>7 ± 6</td>
<td>5 ± 3</td>
<td>0.58</td>
</tr>
</tbody>
</table>

(time elapsed from beginning to cessation of isoflurane administration), and time to extubation (time elapsed from cessation of isoflurane administration until extubation) were recorded for each cat.

Blood sampling and analysis of bupivacaine in plasma

Venous blood samples (1.5 mL) were collected immediately before IP administration of BE or BD (time point 0, baseline) and at 2, 5, 10, 15, 20, 30, 60, 120, 240, 360, and 480 min after administration. These sampling time points were chosen based on findings from previous studies in humans and cats (9,13,14). Hematocrit and total protein were re-evaluated after the last time point to exclude anemia and hypoproteinemia. Samples were collected while the cats were under general anesthesia, with the exception of the following time points: 60, 120, 240, 360, and 480 min. Blood was transferred to EDTA-containing tubes and immediately placed into a container with ice. Samples were kept on ice for 15 to 30 min and then centrifuged at 3500 × g for 10 min. Plasma was separated and stored frozen (−80°C) until analysis.

Pharmacokinetics of bupivacaine

Plasma bupivacaine concentrations were determined using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method as described in a previous study (15). Pharmacokinetic parameters of bupivacaine in cat plasma were calculated using a non-compartmental method (16). The following variables were calculated: area under the plasma concentration-time curve from time zero to the last measured time point (AUC₀-∞, ng h/mL); terminal elimination rate constant (λ₂; 1/h); area under the plasma concentration-time curve from zero (0) hours extrapolated to infinity (∞) (AUC₀-∞, ng h/mL); terminal elimination half-life (T½₂; h); relative clearance indexed by bioavailability (CL/F; L h/kg); and volume of distribution indexed by bioavailability (Vz/F; L/kg).

Postoperative pain and sedation

Analgesia and sedation were evaluated as part of postoperative care. Pain was evaluated by 1 observer (JB), who was not aware of treatment groups, using a multidimensional composite pain scale (UNESP-Botucatu MCPS) (17) and the Glasgow composite feline pain scale (GPS) (18). Pain and sedation were also evaluated using a dynamic visual analog scale (DIVAS pain and DIVAS sedation, respectively) (19) at 60 min before induction of anesthesia (time 0; baseline) and at 0.5, 1, 2, 3, 4, 6, and 8 h after surgery. Rescue analgesia was provided with buprenorphine (0.02 mg/kg IV BW) if MCPS was ≥ 6. Although data collected after the rescue analgesia was administered were not included in the statistical analysis, cats were monitored continuously to determine the requirement for additional analgesia. A second dose of buprenorphine (0.02 mg/kg BW) was administered intramuscularly (IM) to cats at the end of the study or at any time point if needed.

Statistical analysis

Statistical analyses were carried out with standard software (SAS, Version 9.3; SAS Institute, Cary, North Carolina, USA). Data were tested for normality with a Shapiro-Wilk test. Demographic data for each treatment group were analyzed using equal variances t-tests. Plasma drug concentrations and peak plasma drug concentrations-time values were normalized using a log base of 10. Peak plasma-time values were analyzed using an equal-variances t-test. Repeated measures linear models were carried out with time as the within-subject factor and treatment as the between-subject factor, and adjusted with the Benjamini-Hochberg test. The number of cats

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Pharmacokinetic parameters of bupivacaine after intraperitoneal administration of bupivacaine-epinephrine (BE) or bupivacaine-dexmedetomidine (BD) in adult cats undergoing ovariohysterectomy.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group BE</th>
<th>Group BD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{AU}_{\text{C0}} ) (ng h/mL)</td>
<td>7004.3 ± 3302.8</td>
<td>3641 ± 1250.9</td>
</tr>
<tr>
<td>( \text{AU}_{\text{C0}} ) (ng h/mL)</td>
<td>292.2 ± 304.5</td>
<td>25918.5 ± 13539.9</td>
</tr>
<tr>
<td>( \text{C}_{\text{U}} ) (L/kg)</td>
<td>0.148 ± 0.083</td>
<td>0.129 ± 0.069</td>
</tr>
<tr>
<td>( \text{X}_{\text{g}} ) (l/h)</td>
<td>113 ± 10.2</td>
<td>1155 ± 168 ng/mL</td>
</tr>
<tr>
<td>( \text{V/F} ) (L/kg)</td>
<td>1.618 ± 0.906</td>
<td>1.634 ± 0.899</td>
</tr>
<tr>
<td>( \text{t}_{1/2} ) (h)</td>
<td>6.0 ± 2.8</td>
<td>364 3 24</td>
</tr>
</tbody>
</table>

The mean elimination half-life was 11.3 ± 10.2 h (8.9 ± 8.6 h and 10.5 ± 10.3 h; BE and BD groups, respectively). The clearance indexed by bioavailability \((\text{CL/F})\) was 0.148 ± 0.083 L h/kg (0.168 ± 0.069 L h/kg and 0.132 ± 0.129 L h/kg; BE and BD groups, respectively). The volume of distribution indexed by bioavailability \((\text{Vz/F})\) was 1.618 ± 0.906 L/kg (1.634 ± 0.899 L/kg and 1.195 ± 0.777 L/kg; BE and BD groups, respectively). Pharmacokinetic parameters were not different between treatments \((P > 0.05)\).

One cat in the BE group required rescue analgesia at 1 h post-extrusion. An effect of treatment on the number of cats requiring rescue analgesia was rejected \((P = 0.3)\). Pain and sedation scores were not different between treatments \((P > 0.05)\). The MCPS scores were increased in cats of the BE group at 0.5, 1, 2, 3, 4, 6, and 8 h \((P < 0.001\) for all time points) and of the BD group at 0.5, 1, and 2 h \((P < 0.0003\) for all time points) compared with baseline values (Figure 4). The GPS scores were increased in the BE group at 0.5, 1, 2, 3, and 6 h \((P < 0.01\) for all time points) and in the BD group at 0.5, 1, and 2 h \((P < 0.001\) for all time points) when compared with baseline values (Figure 5). Dynamic visual analog scale (DIVAS) pain scores were not significantly different when compared with baseline values. Sedation scores (DIVAS) were significantly increased in the BE group at 0.5 h \((P < 0.0001)\) and in the BD group at 0.5 and 1 h \((P < 0.0001\) for both time points) compared with baseline values.

**Discussion**

Sustained plasma concentrations of bupivacaine were measured after IP administration of BE and BD without adverse effects, as similarly reported in humans (20). Mean maximum plasma concentrations over time (ng/mL) for each group are shown in Figure 1. Individual plasma concentrations over time (ng/mL) are shown in Figure 2 and Figure 3 for the BE and BD groups, respectively. Maximum bupivacaine plasma concentration \((\text{C}_{\text{max}})\) for the BE and BD groups were 1155 ± 168 ng/mL and 1678 ± 364 ng/mL \((P = 0.29)\) at 67 ± 13 min \((\text{TMx})\) and 123 ± 59 min \((P = 0.17)\), respectively.

The dose \((\text{mean ± SD})\) of propofol administered for induction of anesthesia was 10.2 ± 3.1 mg/kg BW \((10.8 ± 3.3\text{ mg/kg BW and 9.7 ± 3.1\text{ mg/kg BW for BE and BD groups, respectively, P > 0.05})\). All cats were discharged from hospital 24 h after surgery without postoperative complications. No signs of local anesthetic toxicity were recorded.

Pharmacokinetic parameters of bupivacaine after IP administration are shown in Table II. Plasma concentrations over time (ng/mL) for each group are shown in Figure 1. Individual plasma concentrations over time (ng/mL) are shown in Figure 2 and Figure 3 for the BE and BD groups, respectively. Maximum bupivacaine plasma concentration \((\text{C}_{\text{max}})\) for the BE and BD groups were 1155 ± 168 ng/mL and 1678 ± 364 ng/mL \((P = 0.29)\) at 67 ± 13 min \((\text{TMx})\) and 123 ± 59 min \((P = 0.17)\), respectively.

The mean elimination half-life was 11.3 ± 10.2 h (8.9 ± 8.6 h and 10.5 ± 10.3 h; BE and BD groups, respectively). The clearance indexed by bioavailability \((\text{CL/F})\) was 0.148 ± 0.083 L h/kg (0.168 ± 0.069 L h/kg and 0.132 ± 0.129 L h/kg; BE and BD groups, respectively). The volume of distribution indexed by bioavailability \((\text{Vz/F})\) was 1.618 ± 0.906 L/kg (1.634 ± 0.899 L/kg and 1.195 ± 0.777 L/kg; BE and BD groups, respectively). Pharmacokinetic parameters were not different between treatments \((P > 0.05)\).
concentrations of bupivacaine were not significantly different between the BE and BD groups and were far from those reported to cause convulsive electroencephalogram pattern (20) or fatal arrhythmias (21) in cats. It is therefore safe to administer these drug combinations in cats undergoing ovariohysterectomy. Both treatments resulted in comparable pharmacokinetics. Interestingly, Cmax (BE − 67 ± 13 min; BD − 123 ± 59 min) and bupivacaine alone (30 ± 24 min) (9). This was particularly true for the BD group versus bupivacaine alone, where a 4-fold difference for Tmax and approximately 50% difference in mean Cmax were detected. These 2 variables (Tmax and Cmax) are influenced by the relationship between absorption, distribution, and elimination rate constants. Since terminal elimination rate constant and clearance indexed by bioavailability were similar between treatments, it is possible that vasoconstriction produced by epinephrine and dexmedetomidine played a role in delayed absorption and longer terminal elimination half-life in the BE and BD groups compared with bupivacaine alone.

The safety and efficacy of IP analgesia depend on many factors, including the use of different local anesthetics or their combinations; doses; concentrations; volumes of injection; mode of administration, (i.e., aerosol, nebulization versus instillation); and addition of other adjuvants such as opioids, etc. Many of these factors have not been investigated in feline medicine and surgery and it is not known how they would change pharmacokinetics and pharmacodynamics of analgesics. Furthermore, modifications of the technique, such as post-incisional versus end of surgery, frequency (single versus multiple doses versus infusions), and site of administration (port-site or directed infiltration versus instillation), could also impact safety and efficacy. Future studies are warranted to investigate these aspects of IP analgesia in feline practice and their effects on pharmacokinetics and pharmacodynamics.

This study had some limitations. Firstly, there was no control group receiving bupivacaine alone. Such a group would have validated our results and highlighted the advantages of using bupivacaine-epinephrine and bupivacaine-dexmedetomidine solutions compared with bupivacaine alone, especially when using a small population of cats with large individual variability. Such a control group was not included in the present study due to financial constraints and because it would have been ethically unacceptable since the pharmacokinetics of bupivacaine after IP administration have already been described in this species (9). Comparisons with
In conclusion, IP bupivacaine with epinephrine or dexmedetomidine produced concentrations below toxic levels and are safe to administer for IP analgesia in cats. Similar postoperative analgesia was observed for bupivacaine-epinephrine and bupivacaine-dexmedetomidine in combination with meloxicam and buprenorphine.

**Acknowledgments**

The authors thank Fleur Gaudette from the Pharmacokinetics core facility of the Centre de Recherche, Centre hospitalier de l’Université de Montréal (CRCHUM) for carrying out LC-MS/MS method development, validation, and sample analysis; Guy Beauchamp for statistical analysis; and Faustine Sarceau for technical help during the study. Funding was provided by the American College of Veterinary Anesthesia and Analgesia (ACVAA) Research Foundation; the “Fonds du Centenaire” of the Faculty of Veterinary Medicine, Université de Montréal; and a generous donation by Valeria Rosenbloom and Mike Rosenbloom. Dr. Beatriz Monteiro is a recipient of the Vanier Canada Graduate Scholarship.

**References**


H9N2 avian influenza virus retained low pathogenicity after serial passage in chickens

Akinlolu Jegede, Qigao Fu, Yohannes Berhane, Min Lin, Ashok Kumar, Jiewen Guan

Abstract

The H9N2 strains of avian influenza viruses (AIVs) circulate worldwide in poultry and cause sporadic infection in humans. To better understand the evolution of these viruses while circulating in poultry, an H9N2 chicken isolate was passaged 19 times in chickens via aerosol inoculation. Whole-genome sequencing showed that the viruses from the initial stock and those after the 8th and 19th passages (P0, P8, and P19) all had the same monobasic cleavage site in the hemagglutinin (HA), typical for viruses of low pathogenicity. However, at position 226 of the HA protein the ratio of glutamine (which favors avian-type receptor binding) to leucine (which favors mammalian-type receptor binding) decreased from 54:46 in P0, to 87:13 in P8, and then 0:100 in P19. In chickens exposed to aerosols of P0, P8, or P19, replication of the viruses was similar and mainly limited to the respiratory tract. None of the infected chickens showed any clinical signs. Over the 19 passages the viruses maintained relatively stable infectivity but gradually lost lethality to chicken embryos. According to the hemagglutination inactivation assay, P8 was slightly and P19 significantly (P < 0.05) less thermostable than P0. Collectively, after 19 passages in chickens the H9N2 AIVs retained low pathogenicity with a positive selection of L226 in the HA. These findings suggest that H9N2 viruses might acquire mammalian specificity after asymptomatic circulation in avian species.

Résumé

Les souches H9N2 du virus de l'influenza aviaire (VIA) circulent mondialement parmi la volaille et causent des infections sporadiques chez l'humain. Afin de mieux comprendre l'évolution de ces virus alors qu'ils circulent parmi la volaille, un isolat H9N2 de poulet a été passé 19 fois chez des poulets via inoculation par aérosol. Le séquençage du génome complet a démontré que les virus de l'approvisionnement initial et ceux après le 8e et le 19e passages (P0, P8, et P19) avaient tous le même site de clivage monobasique dans l'hémagglutinine (HA), typique des virus de faible pathogénicité. Toutefois, à la position 226 de la protéine HA le ratio de glutamine (qui favorise l’adhésion aux récepteurs de type aviaire) à leucine (qui favorise l’adhésion aux récepteurs de type mammalien) a diminué de 54:46 à P0, à 87:13 à P8, et à 0:100 à P19. Chez des poulets exposés à des aérosols provenant de P0, P8, ou P19, la réplication des virus était similaire et principalement limitée au tractus respiratoire. Aucun des poulets infectés n’a montré de signes cliniques. Sur l’ensemble des 19 passages les virus ont maintenu un pouvoir infectant relativement stable mais ont graduellement perdu leur capacité létale pour les embryons de poulets. En fonction des résultats de l’épreuve d’inactivation de l’hémagglutination, P8 était légèrement et P19 significativement (P < 0.05) moins thermostable que P0. De manière globale, après 19 passages chez des poulets les VIA H9N2 ont maintenu une faible pathogénicité avec sélection positive de L226 dans l’HA. Ces résultats suggèrent que les virus H9N2 pourraient acquérir une spécificité mammalienne après circulation asymptomatique dans les espèces aviaires.

Introduction

The H9N2 subtype of avian influenza viruses (AIVs), first isolated from turkeys in the United States in 1966, has become endemic in poultry in many Eurasian countries since the 1990s (1,2). These viruses are grouped into 3 distinct lineages; namely, A/chicken/Beijing/1/94-like (BJ/94-like), A/quail/Hong Kong/G1/97-like (G1-like), and A/duck/Hong Kong/Y439/97 (Y439-like) (3). Recent evidence shows that these lineages are dispersed in North America by migratory birds (4). The H9N2 AIVs infect both avian and mammalian species (5–7). Although multiple viral components contribute to the host range of AIVs, the hemagglutinin (HA) plays a crucial role in infection. The HA mediates viral entry into cells and has receptor-binding and membrane-fusion activity. The receptor-binding domain (RBD), in the HA head region, is responsible for viral attachment to the host cell (8). Mutations in the RBD, such as H155T, H183N, A190V, and Q226L, contributed to the change in receptor-binding preference of H9N2 AIVs from α2,3-linked sialic acid (the avian-type receptor) to α2,6-linked sialic acid (the human-type receptor) (6,9,10). The fusion domain (FD), in the HA stalk region, mediates HA stability and endosomal-membrane fusion after viral endocytosis and is highly linked to influenza host range.
transmission phenotype, and pathogenic potential (8). A mutation of HA2-D46E in the FD increased the stability of an H9N2 virus and contributed to its transmissibility in guinea pigs (11). Although retaining low pathogenicity in poultry, H9N2 AIVs have caused sporadic human infections (7). In addition, the G1-like and the BJ/94-like viruses contributed internal gene segments to the H5N1 human isolate in Hong Kong and to the H7N9 and the H10N8 human isolates in China (12,13). The airborne transmissibility of natural H9N2 poultry isolates in ferrets further demonstrates the pandemic potential of H9N2 AIVs (10). Therefore, it is important to study the evolution of H9N2 AIVs in poultry.

The H9N2 AIVs cause subclinical to mild disease in domestic poultry unless the viral infection is complicated with other background or secondary infection (14,15). However, there are concerns that H9N2 viruses may evolve like some H5 and H7 viruses and become highly pathogenic while circulating in poultry populations. The increase in pathogenicity is a polygenic trait in which the HA gene plays a major role (16). The polybasic cleavage site in highly pathogenic AIVs enables the cleavage of HA by a family of subtilisin-like proteases that are ubiquitously expressed in various organ tissues (16). Hence, highly pathogenic AIVs may cause systemic infection, which is often accompanied by dysregulation of the immune system, organ failure, and a sharp increase in mortality rate in poultry (17). In comparison, the monobasic cleavage site in AIVs of low pathogenicity limits HA cleavage by trypsin-like enzymes that are only secreted in the respiratory and intestinal tracts of birds, thus resulting in mild, localized infection. Early isolates of H9N2 AIVs from Hong Kong and Guangdong poultry markets possessed an R-S-S-R connecting peptide motif. Given that the highly pathogenic H5 and H7 AIVs have the R-X-R/K-R motif (X = a nonbasic residue [1,18]), a single mutation in the nucleotide sequence at the connecting peptide of the H9N2 virus might be sufficient to produce the motif of a highly pathogenic virus. Furthermore, generation of a recombinant highly pathogenic H9N2 virus was possible with insertion of a pair of dibasic amino acids into the HA cleavage site followed by serial passage of the recombinant virus in chickens (19). However, there have been no reports of the isolation of natural highly pathogenic H9N2 AIVs from poultry.

To better understand the evolution of natural H9N2 AIVs while circulating in poultry populations, we conducted serial passage of an H9N2 chicken isolate in chickens, analyzed whole-genome sequences of the initial and passaged viruses, and compared the viral pathogenicity in chicken embryos and chickens.

### Materials and methods

#### Chickens and eggs

All chickens and embryonated chicken eggs (ECEs) used in this study were from a specific-pathogen-free flock of single-comb white Leghorn chickens maintained at the Ottawa Laboratory (Fallowfield), Canadian Food Inspection Agency (CFIA), Ottawa, Ontario. Oropharyngeal and cloacal swabs collected from the chickens tested negative for the presence of AIVs by the real-time reverse-transcription polymerase chain reaction (RT-PCR) assay targeting the matrix gene (20). Experiments and procedures involving the chickens conformed to guidelines established by the Animal Care Committee at the CFIA and under containment level 3 conditions.

#### Virus passages

The H9N2 strain of AIV used for this study, A/chicken/Henan/1/1998, was provided by Drs. Earl Brown and Shuai Wang at the University of Ottawa, Ottawa, Ontario. The virus was isolated from diseased chickens in the Henan province of China in 1998 (21) and cultivated in Madin–Darby canine kidney cells. Culture fluids containing the viruses were passaged twice in ECEs to prepare a stock. This H9N2 stock (designated as P0) was inoculated into 3 chickens no more than 1 wk old via aerosol exposure as described previously (20). The chickens were euthanized 3 d after inoculation. Tracheal and lung tissue specimens were collected and

### Table I. Nucleotide and amino acid substitutions in gene segments of the passaged H9N2 viruses P0, P8, and P19.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nucleotide alterations</th>
<th>Position</th>
<th>Amino acid substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>A/T P8 P19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>G/C</td>
<td>216</td>
<td>Q/L^b</td>
</tr>
<tr>
<td>G</td>
<td>A/C</td>
<td>212</td>
<td>Q/L^b</td>
</tr>
<tr>
<td>A</td>
<td>G/C</td>
<td>212</td>
<td>Q/L^b</td>
</tr>
<tr>
<td>C</td>
<td>T/C</td>
<td>212</td>
<td>Q/L^b</td>
</tr>
<tr>
<td>PB1</td>
<td>A/G</td>
<td>212</td>
<td>Q/L^b</td>
</tr>
<tr>
<td>NA</td>
<td>A/T P8 P19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>G/C</td>
<td>212</td>
<td>Q/L^b</td>
</tr>
<tr>
<td>C</td>
<td>T/C</td>
<td>212</td>
<td>Q/L^b</td>
</tr>
</tbody>
</table>

^a^ In the left-hand and right-hand columns, respectively, the HA amino acids were numbered according to H9 and H3 HA amino acid numbering by Burke and Smith (24).

^b^ The percentages of 194 reads for Q226 and L226 variants were 54% and 46%.

^c^ The percentages of 172 reads for Q226 and L226 variants were 87% and 13%.

^d^ All 151 reads were L226.

^e^ The NA amino acids were numbered according to N2 NA amino acid numbering by Colman, Hoyne, and Lawrence (25).
homogenized in phosphate-buffered saline (PBS), pH 7.2, 0.01 M, at a 1:9 (weight:volume) ratio. The homogenates were centrifuged to remove tissue debris and the supernatants diluted 10-fold with PBS and used for infection of another 3 chickens via aerosol exposure for the next passage. The process was repeated 19 times, and the passaged viruses were designated as P1 to P19. The diluted tissue homogenates were inoculated into 10-day-old ECEs to generate virus stocks for sequencing and for infection of chickens. Passaging was ended at P19 because the viruses from P16 on were not lethal to chicken embryos. For further studies P0, P8, and P19 were selected from the start, middle, and end of passaging.

Sequencing and sequence analysis

By means of the MagMAX-96 Viral Isolation Kit (Thermo Fisher Scientific, Burlington, Ontario) RNA was extracted from stocks of P0, P8, and P19. The extracted RNA was subjected to whole-genome amplification with use of the PathAmp FluA Reagents (Thermo Fisher Scientific). The resulting amplicons were purified and quantitated and libraries were prepared with the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific). The libraries were quantified with the Ion Library Quantitation Kit (Thermo Fisher Scientific) and then amplified by means of the Ion PGM Template OT2 200 Kit (Thermo Fisher Scientific). Finally, sequencing was done with the Ion PGM Hi-Q Sequencing Kit on the Ion PGM System (Thermo Fisher Scientific).

The nucleotide and deduced amino acid sequences of the H9N2 AIVs used in the study and the reference strain, A/duck/Hong Kong/Y280/97 (accession no. AF156376 in GenBank, National Center for Biotechnology Information, Bethesda, Maryland, USA), were aligned with the Clustal W method in the MegAlign program (Lasergene 13.0.2; DNASTAR, Madison, Wisconsin, USA), and the nucleotide sequences of the 8 segments were compared among P0, P8, and P19 and with those of the avian influenza viruses in GenBank by the BLAST search method.

Chicken experiments

Eighty chickens aged 4 wk were divided into 4 groups. Each group of 20 chickens was exposed to virus or PBS aerosols for 15 min as described previously (20). The virus aerosols had been generated from a suspension containing P0, P8, or P19 at concentrations expressed as a 50% embryo infective dose (EID$_{50}$) of $6.5 \times 10^7$/mL.
After exposure, the chickens were placed in a self-contained isolator that was operated under negative pressure. Feed and water were provided ad libitum. To monitor virus shedding, oropharyngeal and cloacal swabs were collected from 20, 15, 10, and 5 chickens 1, 2, 4, and 7 d after exposure, respectively. To study virus replication and tissue tropism, 5 chickens from each group were randomly selected for euthanasia after collection of swab samples on days 1, 2, 4, and 7 after exposure. Trachea, lung, spleen, liver, small intestine, cecum, tonsil, and bursa tissue specimens were collected from each chicken; each specimen weighing approximately 50 mg. Virus titer in swabs and tissue specimens was determined by means of real-time RT-PCR targeting the matrix gene and was expressed as EID_{50} per milliliter or per gram (20). A 1-way analysis of variance (ANOVA) in XLSTAT software (Addinsoft, Paris, France) for Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) was used to determine significant differences in virus titers in each type of sample and specimen collected on each day after exposure to P0, P8, or P19 among the groups of chickens. The critical level for significance was set at \( P < 0.05 \).

**Pathogenicity in chicken embryos**

Homogenates of the tracheal and lung tissues from chickens that were used for passaging of the H9N2 virus were serially diluted 10-fold. Each dilution was inoculated into five 10-day-old ECEs to determine the titers of the viruses in the homogenates as described by Alexander (22). Allantoic fluids were recovered from the inoculated ECEs after 7 d of incubation or within 24 h after the death of the embryos and were subjected to the hemagglutination assay for detection of influenza virus (22). The EID_{50} and the 50% embryo lethal dose (ELD_{50}) were determined and used as indicators for the pathogenicity of the viruses in chicken embryos.

**Hemagglutination inactivation assay**

Allantoic fluids that contained 256 HA units of P0, P8, and P19 were incubated at 54°C for up to 8 h. The HA titers were measured at a number of time points: 0, 0.25, 0.5, 1, 2, 4, 6, and 8 h. At each time point triplicate samples were retrieved and subjected to the hemagglutination assay (22). The time required to produce a 6-log reduction in HA units (from 256 to 4) was determined with the XLSTAT software and a linear regression model representing the thermal inactivation kinetics as in the equation

\[
y = ax + b
\]

where: \( y \) is the virus HA titer (log_{2}), \( x \) is the time of incubation (h), \( a \) is the slope, and \( b \) is the \( y \)-intercept. The model was considered fit when its coefficient of determination \( R^2 \) exceeded 0.95 and its F test for the linearity of regression was significant at \( P < 0.05 \). With the XLSTAT software the analysis of covariance (23) was used to determine significant differences in the thermal inactivation kinetics or in thermostability between each of the H9N2 viruses. The critical level for significance was set at \( P < 0.05 \).

**Results**

The A/chicken/Henan/1/1998 H9N2 virus used in this study, derived from the BJ/94-like lineage, has an HA gene 99.5% homologous in nucleotides to that of the A/chicken/Hubei/01/1999 H9N2 virus (data not shown). After 19 passages in chickens the H9N2 viruses retained sequence characteristics of low pathogenicity, as revealed by the whole-genome sequence analysis of viruses from the initial stock and the 8th and 19th passages: P0, P8, and P19 all
Supplementary Figure. Amino acid sequence alignment of the HA1 and HA2 subunits of H3 (A/Aichi/2/68; GenBank accession no. J02090), wild-type H9N2 (A/chicken/Henan/1/1998) stock (P0) and passages 8 and 19 (P8 and P19), and H9N2 (Y280) (A/duck/Hong Kong/Y280/97; GenBank accession no. AF156376) avian influenza viruses. Mutations are highlighted in red and the cleavage site is highlighted in yellow. Numbering is according to the H3 numbering system (24). Horizontal lines above sequences indicate respective subdomains (red for fusion subdomains, yellow for vestigial esterase, and blue for receptor binding). Multiple sequence alignment was generated in sequence format with the T-Coffee and Boxshade server.
had an R-S-S-R amino acid motif at the HA cleavage site, which is typical for viruses of low pathogenicity. The P0 and P8 viruses were heterogeneous at position 226 of the HA protein: in P0, 54% of 194 reads were glutamine (Q) and 46% were leucine (L), whereas in P8 the Q to L ratio was 87:13 in 172 reads, and in P19 100% of 151 reads were L (Table I). Four other mutations occurred in the HA protein; namely, N304D, V318I, and H397Y in both P8 and P19, and D375N in P19 (Table I). An N76S mutation occurred in the PB1 protein in both P8 and P19, and an S311R mutation occurred in the neuraminidase protein in P19 (Table I).

None of the 4-week-old chickens showed any clinical signs within 7d after exposure to aerosols of P0, P8, or P19. In general, the viruses had similar replication and tissue tropism in the exposed chickens: P0, P8, and P19 mainly replicated in the respiratory tract, and the titers were greatest on day 1 after exposure, then gradually decreased over the 7d of the experiment (Figure 1). In oropharyngeal swabs the titer of P19 was significantly greater than that of P0 or P8 on day 1 (Figure 1A), but P19 replicated to significantly lower titers in tracheal tissues on day 2 (Figure 1B) and in lung tissues on day 4 compared with P0 (Figure 1C). During the 7-day experiment P0, P8, and P19 were detected only at low titers (< 4.4 log10 EID50/g) in spleen, liver, small intestine, cecum, tonsil, and bursa tissues and were rarely detected at low titers in cloacal swabs (data not shown).

Passage of the H9N2 viruses in chickens decreased the pathogenicity of the viruses in chicken embryos. The viruses from all passages were infective to chicken embryos. The EID50 of all the viruses in chicken embryos (29,30) generated viruses with enhanced replication and transmission of H9N2 viruses in ferrets and guinea pigs (10,11,32,33). However, in this study the positive selection of either Q or L at position 226 and the similar replication of P0, P8, and P19 in chickens suggested that Q226 and L226 might be equivalent in determining the tissue tropism of H9N2 AIVs in chickens, which have both avian-type and human-type receptors expressed in most of their organ tissues (34). On the other hand, the different combinations of amino acid substitutions in the HA protein may influence the selection of Q or L at position 226, especially the D375N (HA2-D46N) substitution in P19, as a D375E substitution enhanced receptor binding of a H9N2 virus in guinea pigs (11). Although the mechanism for the final complete Q226L substitution is unclear, the present results provide direct evidence of positive selection of L226 in the HA during circulation of H9N2 AIVs in poultry. In support of our finding, L226 has become more and more prevalent in the HA of natural H9N2 chicken isolates in Eurasia (6,35,36). In addition, the Q226L substitution that we observed might play a role in the reduction of HA thermostability in P8 and P19, as such substitution contributed to decreased HA stability in a ferret-adapted H5 virus (37). As a surrogate measure of HA stability, HA thermostability is strongly related to HA acid stability or the pH threshold at which endosomal membrane fusion is induced during virus entry (8). Many amino acid residues throughout the HA sequence are involved in the conformational change of HA during the fusion process and influence HA stability (38). In this study, the N304D, V318I, D375N, and H397Y substitutions might also have contributed

Furthermore, passing the H9N2 virus in chickens in this study reduced the pathogenicity but not the infectivity of the virus in chicken embryos. In agreement with our findings, low-pathogenicity H5 AIVs of both wild bird and chicken origin retained low pathogenicity after 6 passages in chickens (26). In addition, passing influenza viruses in chicken embryos has been commonly used to attenuate a virus for vaccine development (27,28). However, in contrast to our findings, passing H9N2 AIVs in chickens (19) or in chicken embryos (29,30) generated viruses with enhanced replication and pathogenicity in both chicken embryos and chickens. An H5 wild swan virus of low pathogenicity became highly pathogenic in chickens after 24 passages by air sac inoculation followed by 5 passages in chicken brains (31). In addition, serial passages in quails and chickens enabled a duck H9N2 virus to readily infect mice (32). As suggested by Dlugosinski et al (26), genetic changes are not predictable during AIV passage in animals and may result in various phenotypes.

In this study, single-point amino acid selection and mutations occurred in the HA gene and might have had impacts on host receptor-binding specificity and HA stability. The parent H9N2 AIV (P0), a chicken isolate, contained 2 almost equal virus subpopulations or quasispecies with Q or L at position 226 of the HA protein. During passage in chickens Q226 was positively selected over L226 early on (P8) and then completely substituted by L226 later (P19). In comparison, an L226Q substitution was observed during passage of a recombinant H9N2 virus in chickens by Soda et al (19). The Q226L substitution in the HA is well known for confering avian H9N2 viruses with the ability to bind to human-type receptors and to replicate efficiently in human airway epithelial cells (5,6,9–11). In addition, L226 played an important role in the replication and transmission of H9N2 viruses in ferrets and guinea pigs (10,11,32,33). However, in this study the positive selection of either Q or L at position 226 and the similar replication of P0, P8, and P19 in chickens suggested that Q226 and L226 might be equivalent in determining the tissue tropism of H9N2 AIVs in chickens, which have both avian-type and human-type receptors expressed in most of their organ tissues (34). On the other hand, the different combinations of amino acid substitutions in the HA protein may influence the selection of Q or L at position 226, especially the D375N (HA2-D46N) substitution in P19, as a D375E substitution enhanced receptor binding of a H9N2 virus in guinea pigs (11). Although the mechanism for the final complete Q226L substitution is unclear, the present results provide direct evidence of positive selection of L226 in the HA during circulation of H9N2 AIVs in poultry. In support of our finding, L226 has become more and more prevalent in the HA of natural H9N2 chicken isolates in Eurasia (6,35,36). In addition, the Q226L substitution that we observed might play a role in the reduction of HA thermostability in P8 and P19, as such substitution contributed to decreased HA stability in a ferret-adapted H5 virus (37). As a surrogate measure of HA stability, HA thermostability is strongly related to HA acid stability or the pH threshold at which endosomal membrane fusion is induced during virus entry (8). Many amino acid residues throughout the HA sequence are involved in the conformational change of HA during the fusion process and influence HA stability (38). In this study, the N304D, V318I, D375N, and H397Y substitutions might also have contributed
to the change in HA thermostability, as these amino acid residues are located close to the HA subunit interface in the fusion domain. Previous studies found that a T318I substitution stabilized the HA in an H5 virus during adaptation in ferrets (37), and a D375E (HA2-D46E) substitution improved HA thermostability, enhanced receptor binding, and contributed to the contact transmission of an H9N2 virus in guinea pigs (11). The results of these adaptation studies suggested that HA stability is correlated with host specificity, pathogenicity, and transmissibility of influenza viruses. Stabilized HAs were required for efficient transmission of H5N1 or H9N2 viruses among mammalian species (11,37). Additionally, most HAs from human isolates were more stable than those from avian isolates of the same subtype (39). In contrast, a decrease in HA stability was associated with an increase in the pathogenicity of H5N1 viruses in chickens (40). However, passing in chickens in our study decreased HA thermostability in the H9N2 virus and reduced its pathogenicity in chicken embryos.

In conclusion, this study has provided evidence of positive selection of L226 in the HA and stable low pathogenicity of the H9N2 AIVs after serial passage in chickens. These results suggest that the viruses might acquire mammalian specificity after asymptomatic circulation in avian species.

Acknowledgments

This study was funded by the Canadian Food Inspection Agency (CFIA). The authors thank Drs. Earl Brown and Shuai Wang at the University of Ottawa for providing the H9N2 avian influenza virus strain; Dr. Susan Nadin-Davis, Dr. Marc-Olivier Duceppe, and Mr. Bradley Pickering at the CFIA for providing support in sequencing the viruses and analyzing the sequence information; and the animal care staff at the CFIA for assisting with the animal work.

References

Time course of Salmonella shedding and antibody response in naturally infected pigs during grower-finisher stage

Saranya Nair, Abdolvahab Farzan, Terry L. O’Sullivan, Robert M. Friendship

Abstract

A longitudinal trial was conducted to determine the course of Salmonella shedding and antibody response in naturally infected grower-finisher pigs. Ten-week-old pigs (n = 45) were transferred from a farm with history of salmonellosis and housed at a research facility. Weekly fecal samples (weeks 1 to 11) as well as tissue samples at slaughter were cultured for Salmonella. Serum samples were tested for presence of Salmonella antibody by enzyme-linked immunosorbent assay (ELISA). Data were analyzed using a multilevel mixed-effects logistic regression model. Over 10 wk, 91% and 9% of pigs shed Salmonella ≤ 4 and > 5 times, respectively. The estimated median of Salmonella shedding duration was 3 to 4 wk but some pigs shed Salmonella for up to 8 wk. Salmonella shedding increased 1 wk post-arrival but followed a decreasing pattern afterwards up to week 11 (P < 0.05). Salmonella isolates (n = 29), which were recovered from 18 pigs at different occasions, were S. Typhimurium (28%), S. Livingstone (21%), S. Infantis (14%), S. Montevideo (7%), S. Benfica (3%), S. Amsterdam (3%), S. Senftenberg (17%), and S. I.Rough-O (7%). Of 11 pigs from which the first and last isolates were serotyped, 10 pigs were reinfected with a different serotype. At slaughter, Salmonella was isolated from 7 pigs, of which 5 (71%) had not tested positive for at least 7 wk prior to slaughter. Antibody response peaked 4 wk after the peak of Salmonella infection; Salmonella shedding reduced as antibody response elevated (P < 0.05). These findings indicate that pigs may shed Salmonella into the mid-point of the grower-finisher stage and may be reinfected with different serotypes.

Résumé

Un essai longitudinal a été réalisé afin de déterminer la progression de l’excrétion de Salmonella et la réponse en anticorps chez des porcs en période de croissance-finition naturellement infectés. Des porcs âgés de 10 semaines (n = 45) ont été transférés d’une ferme avec une histoire de salmonellose et hébergés dans une installation de recherche. Des échantillons de fèces ont été prélevés à chaque semaine (semaines 1 à 11) de même que des échantillons de tissus lors de l’abattage et ont été cultivés pour Salmonella. Des échantillons de sérum ont été testés pour la présence d’anticorps contre Salmonella par une épreuve immunoenzymatique (ELISA). Les résultats ont été analysés par un modèle de régression logistique multiniveaux à effets mixtes. Au-delà de 10 semaines, 91 % et 9 % des porcs excréraient Salmonella ≤ 4 et > 5 fois, respectivement. La médiane estimée de la durée d’excrétion de Salmonella était de 3 à 4 sem mais quelques porcs ont excrété Salmonella jusqu’à 8 sem. L’excrétion de Salmonella augmenta 1 sem après l’arrivée mais fut suivie par la suite d’un patron de diminution jusqu’à la semaine 11 (P < 0.05). Les isolats de Salmonella (n = 29) qui ont été obtenus de 18 porcs à différentes occasions, étaient S. Typhimurium (28 %), S. Livingstone (21 %), S. Infantis (14 %), S. Montevideo (7 %), S. Benfica (3 %), S. Amsterdam (3 %), S. Senftenberg (17 %) et S. I :Rough-O (7 %). De 11 porcs pour lesquels les premiers et les derniers isolats furent serotyped, 10 porcs étaient réinfectés avec un sérotype différent. Lors de l’abattage, Salmonella a été isolé de sept porcs, parmi lesquels cinq (71 %) n’avaient pas eu de culture positive pour au moins 7 sem avant l’abattage. La réponse en anticorps a présenté un pic 4 sem après le pic d’infection par Salmonella; l’excrétion de Salmonella a diminué alors que la réponse en anticorps augmentait (P < 0.05). Ces résultats indiquent que les porcs peuvent excrêter Salmonella jusqu’au milieu de leur période de croissance-finition et peuvent être réinfectés avec un sérotype différent.

Introduction

The emergence of non-typhoidal Salmonella spp. (Salmonella), in particular multi-antimicrobial resistant strains, presents a public health and food safety concern. The spread of Salmonella to humans can occur through consumption of contaminated pork products. However, manure from pig farms also poses a threat because of the risk of water contamination and the presence of Salmonella on produce where manure is used as a fertilizer. Understanding the dynamics and epidemiology of Salmonella infection in the swine population is vital in improving prevention and control of Salmonella at the farm level and reducing the “farm-to-fork” transmission of the pathogen.

The prevalence of Salmonella shedding has been reported to be highest in the nursery stage and decline during the grower-finisher period until slaughter (1,2). However, various longitudinal studies have reported variability in Salmonella shedding patterns and have detected a higher than expected prevalence of Salmonella shedding in the grower-finisher stage (3,4). Furthermore, the distribution of
serotypes can also greatly impact the duration of Salmonella shedding (3,5) and affect whether or not Salmonella is recovered from tissue at slaughter (6).

Due to the lack of clinical signs in asymptomatic carriers, it is difficult to identify Salmonella infection in pigs. Serological testing methods, which assess antibody response to Salmonella infection, have been found to be more effective in capturing the population of intermittent shedders and the history of exposure to Salmonella than traditional bacteriological methods. Research has revealed that a pig’s passive immunity to Salmonella may last less than 8 wk of age, but its active immunity in response to Salmonella infection may be present before 8 to 9 wk of age (7). However, variability in the age of onset of infection to the time of host antibody response has been noted (8). Studies have also reported a delayed onset of serological response (7) and a delayed peak in seropositivity from the peak in Salmonella infection (1).

The objectives of this study were: i) to investigate the Salmonella shedding pattern in naturally infected pigs through the grower-finisher stage, ii) to examine the development of antibody levels in relation to the Salmonella shedding pattern, and iii) to determine the presence of Salmonella in tissue and cecal content at slaughter.

**Materials and methods**

The research trial was approved by the University of Guelph Animal Care Committee, in accordance with the guidelines set forward by the Canadian Council of Animal Care.

**Pigs**

Prior to the start of the study, a commercial swine farm in Ontario with a history of high Salmonella prevalence was identified and visited. Salmonella was recovered from gestation sow stalls and nursery pens on this operation. Forty-five naturally infected 10-week-old pigs weighing approximately 25 kg from this farm were transported to Ponsonby General Animal Facility, a research facility at the University of Guelph. Upon arrival (week 1), the pigs were ear tagged for individual identification and randomly assigned to pens on this operation. Forty-five naturally infected 10-week-old pigs were sent to slaughter at weeks 13 or 14 (at 22 to 23 wk of age). Animals were transported in small groups of about 15 and housed individually during the short waiting time (< 1 h) from arrival until slaughter. At slaughter, tissue samples from the liver, spleen, neck lymph node, ileoceleal lymph node, and tonsil as well as cecal contents were collected from each pig. All samples were transported to the laboratory in insulated coolers with ice packs.

**Salmonella isolation**

The fecal and tissue samples were diluted with Tetrathionate Broth (TTB) (Becton Dickinson, Franklin Lakes, New Jersey, USA) at a 1 to 9 ratio. The samples were homogenized for 30 s (1 min for tissue samples) with a Seward Stomacher 400 Circulator (Seward Laboratory Systems) and incubated at 37°C for 18 to 24 h. Then, 100 μL of TTB was transferred to 9.9 mL of Rappaport-Vassiliadis (RV) Broth (Becton Dickinson) and incubated at 41°C for 18 to 24 h. Next, a loopful (20 mL) of the RV Broth was streaked onto xylose-lysine-tergitol 4 (XLT-4) agar plates (Remel XLT-4 Agar; Thermo Fisher Scientific, Lenexa, Kansas, USA) and incubated at 37°C for 18 to 24 h. The suspected Salmonella colonies were tested by an agglutination test using a Salmonella poly O antisera (Becton Dickinson).

**Salmonella serogrouping and serotyping**

The Salmonella isolates (n = 89) from the first and last shedding per pig and the isolates recovered from tissue samples were serogrouped by agglutination slide test using BD antisera (Becton, Dickinson and Company). In addition, a subset of these isolates (n = 29) was submitted to Biovet (St-Hyacinthe, Quebec) for molecular serotyping by means of xMAP Salmonella Serotyping Assay Kit, a microsphere-based method that detects genes that express serotype-specific O and H antigens. The subset of isolates included the first shedding (n = 11) and last shedding (n = 13) for 13 pigs as well as the isolates recovered from tissue samples (n = 5) from 5 other pigs.

**Salmonella antibody detection**

Serum samples were assessed for presence of antibodies using an indirect enzyme-linked immunosorbent assay (ELISA) pigtype Salmonella Ab kit (QIAGEN Leipzig GmbH, Leipzig, Germany). The ELISA kit detects antibodies to Salmonella serogroups B, C, D, and E (O-antigens 1, 3, 4, 5, 6, 7, 9, 10, and 12). The ELISA was performed as described by the manufacturer’s instructions. The optical density (OD) values were used to calculate the sample to positive (S/P) ratio using the following equation:

\[
S/P = \frac{(OD_{sample} - OD_{negative\ control})}{(OD_{positive\ control} - OD_{negative\ control})}
\]
A pig was identified as seropositive if the S/P ratio was > 0.3 and seronegative if it was < 0.3.

**Data analysis**

Data were entered into Microsoft Excel for Mac 2011 Version 14.5.5 (Microsoft, Redmond, Washington, USA) and then imported into Stata (Stata/SE 14.1 for Mac; StataCorp, College Station, Texas, USA). A Kaplan-Meier survival function was used to present and evaluate *Salmonella* shedding over time. The time to event was identified as the last time a pig shed *Salmonella*. In addition, a multilevel mixed-effects logistic regression model with “pen” (common environment) and “pig” (repeated measurements from week 1 to week 11) as random effect was used to analyze the prevalence of *Salmonella* shedding over time. “Age” and “room” variables were included as fixed effect; “room” variable was included as a fixed effect in order to control for the addition of a second room in week 8 to week 11. In addition, a multilevel mixed-effects logistic regression model with “pen” and “pig” as random effect and “room” as a fixed effect was used to determine the association of antibody response (seropositivity) with *Salmonella* shedding.

**Results**

**Salmonella shedding**

Two pigs were euthanized in week 3 for reasons unrelated to the trial. A total of 479 individual fecal samples were collected over the 10-week duration of the trial. All pigs tested positive for *Salmonella* shedding at least once, with 89% of pigs testing positive more than once (Figure 1). *Salmonella* could be recovered between 1 to 8 times from fecal samples collected from each pig weekly. Out of the 45 pigs, 41 (91%) pigs were positive 4 times or less and 4 (9%) pigs tested positive 5 times or more over the 10-week trial (Figure 1). *Salmonella* was recovered from 36 (80%) pigs at arrival to the research facility (week 1) (Figure 2). The remaining 9 (20%) pigs were positive at week 2 or week 3 (Figure 1). *Salmonella* shedding declined in week 2 (38%) but increased in week 3 (91%). After week 3, the number of pigs shedding *Salmonella* decreased but never achieved 0. The overall mean prevalence of *Salmonella* shedding was 27% from week 1 to week 11.

The Kaplan-Meier survival function revealed a 50% survival probability (i.e., pigs stop shedding *Salmonella*) at week 3 and an 80% survival probability at week 6 (Figure 3). However, the survival curve shows *Salmonella* shedding until the end of the trial, with 90% survival probability at week 9 and *Salmonella* being recovered from 2 (5%) pigs at week 11 (Figure 3).

Using the survival analysis data of when pigs started shedding (i.e., first culture-positive) and when they stopped shedding (i.e., last culture-positive), the duration of *Salmonella* shedding was estimated. The estimations were based on the assumptions that a pig was shedding for a week prior to the first isolation and for a week after the last isolation (1). Based on these assumptions, the median time of shedding was 3 to 4 wk. The longest duration of *Salmonella* shedding was estimated to be 3 or 4 wk (17 pigs; 37.8%) and 4 or 5 wk (10 pigs; 22.2%). The range of the *Salmonella* shedding period was from 1 to 12 wk, with 2 pigs shedding *Salmonella* for the entire duration of the study.

When analyzing *Salmonella* shedding over time using a multilevel mixed-effects logistic regression method, the addition of the second room at week 8 was found to have no impact. Due to the lack of linearity, age was modeled as ordinal data. The analysis revealed that as pigs aged from week 2 to week 3, the odds of *Salmonella* shedding was 18.5 times greater [95% CI (2.85, 119)] in comparison to pigs from week 1 to week 2 (P = 0.002). Whereas, from week 3 to week 11, there was a significant decrease in *Salmonella* shedding in comparison to pigs from week 2 to week 3 [OR = 0.002, 95% CI (0.00, 0.01), P = 0.001].

**Salmonella antibody**

Figure 4 illustrates the serological prevalence (serum adjusted OD) at weeks 1, 4, 7, and 11 using boxplots. The mean level of adjusted OD in pigs is shown in Figure 3. Multilevel mixed effects analysis revealed *Salmonella*-shedder pigs are less likely to be tested seropositive by ELISA than non-shedder pigs [OR = 0.38, 95% CI (0.15, 0.98), P = 0.04] when taking into account repeated measures at the pig and pen level over weeks 1 to 11. Furthermore, the peak of *Salmonella* seroprevalence was at week 7, which was 4 wk after the peak in shedding (week 3) (Figure 3).

**Presence of Salmonella in tissue samples**

At slaughter, 254 samples were collected from 43 pigs and cultured for *Salmonella*. Overall, 16% (7/43) of pigs tested positive for *Salmonella* in at least 1 or more tissues or cecal content culture; 1 pig tested positive for 2 tissue samples. The mean prevalence of *Salmonella* in tissue samples was 3.1% (8/254). *Salmonella* was only recovered from 1 ileocecal lymph node, 1 neck lymph node, 1 spleen, 1 tonsil, and 1 liver but from 3 cecal content samples.

The majority of pigs [71% (5/7)] harboring *Salmonella* at slaughter had tested negative on fecal culture for 7 wk or longer prior to slaughter (Figure 1). When assessing the antibody levels in pigs that were *Salmonella* positive at slaughter, 2 (29%) pigs were found seronegative on one or more occasions. The first pig, which was *Salmonella* positive at week 3, was found to be seronegative at week 11 but S. Infantis positive at slaughter. While the second pig, which was *Salmonella* positive at week 4, was found to be seronegative over the duration of the entire trial but S. Typhimurium positive at slaughter.

**Salmonella serogroups and serotypes**

Eighty-nine isolates recovered from 37 pigs at different occasions belonged to serogroups C1 (44%), E4 (29%), B1 (19%), and others (8%). Furthermore, 70.3% (26/37) of pigs were reinfected with *Salmonella* belonging to a different serogroup during the study period; from serogroup E4 to serogroup C1 (37%; 13/37) and then to serogroup B (11%; 4/37), was the most frequent reinfection pattern. Most reinfection patterns were seen in week 3 and week 4. In addition, pigs shedding serogroup C1 (14%; 4/37) were not reinfected with other serogroups. Twenty-nine isolates recovered from 18 pigs at different occasions were serotyped. In total, pigs shed 8 different serotypes over the study period including S. Typhimurium (28%), S. Livingstone (21%), S. Infantis (14%), S. Montevideo (7%), S. Benfica (3%), S. Amsterdam (3%), S. Senftenberg (17%), and S. I:Rough-O (7%). The isolates recovered from tissue samples were
S. Infantis and S. Typhimurium. Of the 11 pigs serotyped for first and last isolates, 91% (10) were reinfected with a different serotype. Pigs were initially colonized by S. Typhimurium (36%), S. Senftenberg (36%), S. Benfica (9%), S. Amsterdam (9%), and S. I:Rough-O (9%); however, reinfection with S. Livingstone (45%) was the most common followed by S. Typhimurium (18%), S. Montevideo (18%), S. Senftenberg (9%), and S. I:Rough-O (9%).

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Figure 1. This chart illustrates the Salmonella status (determined by bacterial culturing) in grower-finisher pigs from the beginning of the trial at week 1 (W1; 10 wk of age) to week 11 (W11; 20 wk of age) and at slaughter (SH; 22 or 23 wk of age) based on how many times the pig was positive (counts) by trend of Salmonella shedding grouped together. The sub-column underneath count data reveals the number of pigs with the same Salmonella shedding pattern within that Salmonella positive count. Grey squares represent Salmonella positive and white squares represent Salmonella negative.

Discussion

Although appearing clinically healthy and coming from a single source, pigs included in this study had a high prevalence of Salmonella shedding and were infected with multiple serotypes of Salmonella. In particular, 4 trends were observed in the time course of Salmonella shedding: i) high prevalence of Salmonella shedding...
at entry, ii) decline of Salmonella shedding, iii) peak in Salmonella shedding, and iv) gradual decline in Salmonella shedding. Although most pigs stopped shedding during the finisher phase, Salmonella positivity status in the group was maintained due to a few pigs that continued to shed Salmonella throughout the finisher phase.

Younger pigs tend to have increased Salmonella shedding (9) as a result of being more susceptible to disease and infection due to a compromised immune system (10,11). The increased potency of Salmonella serotypes may also play a role in establishing permanent infections (5). In the present trial, most of the pigs were infected at entry into the grower-finisher period (10 wk of age) and some were found to shed Salmonella for up to 8 wk. It is almost certain that the pigs in this study had already been colonized by Salmonella during the nursery stage and the stress of transport may have caused an increase in shedding resulting in a high prevalence of Salmonella shedding in pigs at entry (2,9). This is supported by research that suggests that stress, particularly an increase in cortisol experienced during transport, intensifies the colonization of Salmonella (12).

Although pigs are less likely to shed Salmonella as they mature, the variability in Salmonella prevalence and shedding pattern during the early grower phase may be a result of reinfection and renewed Salmonella shedding caused by the presence of multiple serotypes, most of which have been previously reported on Ontario swine farms (13,14). Findings of this current study indicate that the 4 trends witnessed were most likely a result of pigs experiencing infection with serotypes of Salmonella different from the ones they had previously been colonized with. In particular, S. Livingstone, most commonly found to cause reinfection in pigs in the present study, has been found to have rapid transmission (15).

While most of the pigs were reinfected with different serotypes, there were a number of pigs that remained Salmonella positive with the same serotype. This introduces the possibility of cross protection amongst some of the serotypes that colonized pigs in the current study. For example, pigs that were initially infected with serogroup C1 were not infected with any other serogroup. However, the observation that most pigs were reinfected with different serotypes indicates the lack of cross-protection among some serotypes. The failure of exposure to one serotype to protect against colonization of a different Salmonella serotype has been previously reported (15).

Over time, as the pigs progressed through the grower-finisher period, there was an overall reduction in Salmonella shedding. Low levels of Salmonella before slaughter, which were found herein, have also been reported in other longitudinal studies (1–3,16). Many farm prevalence studies have used fecal cultures of pigs close to market weight. This study demonstrates that by sampling these older animals, the true on-farm prevalence may appear lower than it actually is. This has implications with regards to monitoring programs. In a herd with a shedding pattern such as the one observed in this study, if pigs are only monitored in the late stages of the finishing barn, one might get the impression that there is no or very little Salmonella present in the herd. Evaluating Salmonella herd health on the basis of Salmonella status in late finisher stage is not recommended as pigs are likely asymptomatic.

In the present study, following the low levels of Salmonella in the late finisher stage, S. Typhimurium and S. Infantis were recovered at slaughter. These findings are consistent with what has been reported in tissue and cecal content at slaughter in earlier studies (6,17,18). Pigs infected with S. Typhimurium are asymptomatic carriers that are atypically colonized by Salmonella in the tonsils, guts, and gut-associated lymph nodes for weeks or months (19) with little to no shedding of Salmonella prior to slaughter (20). However, 2 of the chronic shedders in this study were infected with S. Typhimurium and the strain was recovered from fecal samples toward the end of the trial and from tissue at slaughter. Whereas S. Livingstone, which was frequently recovered from pigs in this study, was not isolated at slaughter. Further research assessing Salmonella serotypes can be helpful in understanding which strains cause prolonged Salmonella shedding and which are more prone to colonize tissue.

In contrast to the bacteriology results, which revealed a gradual decline in Salmonella shedding, seropositivity was found to increase toward the end of finishing. At arrival to the research facility, this population of pigs was found to have an 80% prevalence of Salmonella shedding, whereas they had a seropositivity of less than 40%. Research has suggested that the persistence of maternal antibodies is typically less than 8 wk, while active immunity can
develop before 8 to 9 wk of age (7). This leads us to believe that the maternal antibodies had diminished and the pigs had yet to mount active immunity against *Salmonella* infection when the first samples were analyzed.

After the peak in *Salmonella* shedding at week 3, there was a 4-week delay until the peak in antibody response was observed. The low seropositivity witnessed at arrival and the delayed onset of antibody response could in part be due to re-infection by different serotypes of *Salmonella*. Although the pigs might have antibodies against some serotypes, they still became infected with new serotypes. There is a possibility that the antibody response to certain serotypes may protect against other serotypes from the same serogroup.

Previous findings from a longitudinal study, conducted in 3 farrow-to-finish swine herds in 2 cohorts using a total of 180 pigs with a *S. Typhimurium* infection, reported a peak in seroprevalence in mid-finishing stage at 17 wk of age with the antibody response approximately 60 d (8 to 9 wk) after the peak in *Salmonella* shedding (1). A similar peak in seroprevalence was noted in the present study; however, the antibody response occurred much sooner after the peak in *Salmonella* shedding. Whereas, another longitudinal study of *Salmonella* shedding in a farrow-to-finish farm found a delayed onset of *Salmonella* seropositivity until the last third of the finishing phase with *Salmonella* shedding, predominantly in the first half of the grower-finisher stage (7). In contrast, an experimental challenge study reported that pigs infected with *S. Typhimurium* became seropositive at day 7, peaking 30 d post-inoculation and with variability in the time of seropositivity and the persistence of the antibody response amongst pigs (8).

The time from the peak in *Salmonella* infection to the peak in antibody response varies from study to study. This may also be a result of differences in study design, data collection, and/or testing methods. The difference in serotypes present on farms or the serotypes pigs are challenged with may have a great impact on not just the *Salmonella* shedding but antibody response.

The present study found that the majority of pigs that were *Salmonella* positive at slaughter were also seropositive, although they were considered *Salmonella* negative on the basis of bacterial culture of their feces. Similarly, Nielsen et al (8) found that *S. Typhimurium* challenged pigs that had been bacterial culture-negative for 65 to 106 d but culture-positive at slaughter were also seropositive. In the present study, *S. Typhimurium* and *S. Infantis* were commonly recovered at slaughter from intermittent carriers that had been *Salmonella* culture-negative for over 7 wk. The serological testing methods may be able to identify pigs that are colonized by *Salmonella* when bacterial culture is not able to identify these asymptomatic *Salmonella*-carrier pigs (21). However, 1 pig infected with *S. Typhimurium* at slaughter, was found to be seronegative over the span of the study. Future research may explore immunological tolerance between pig and *Salmonella* serotype.

The present study, with a population of pigs infected with multiple serotypes, is a good representation of a farm that is highly infected with *Salmonella*. Findings from the present study and previously published studies reveal variability in *Salmonella* shedding, duration, colonization in tissue, and antibody response to *Salmonella*, which may be due to the differences in sampling methods, virulence of different serotypes, genetics of pigs, and husbandry practices (8,22). In the present study, pigs were tested weekly by *Salmonella* fecal culturing and in 3- to 4-week intervals by ELISA for serological response to *Salmonella*. Bacteriology testing was predominately conducted using fecal samples and rarely using rectal swabs, as previous studies have reported a lower sensitivity in detecting *Salmonella* when culturing from rectal swabs (23,24). Although weekly fecal culture testing increased the probability of *Salmonella* detection, the study is limited to a small population of pigs from a single source and the findings may not be extrapolated to a broad population or compared to prevalence studies. In addition, understanding of the association between *Salmonella* shedding and *Salmonella* antibody response is restricted because fluctuations in antibody response were not captured during the 3- to 4-week intervals between serological testing. Furthermore, the present study cannot be compared to many challenge studies that assess the prevalence of *Salmonella* shedding and antibody response to *Salmonella* in pigs colonized by one serotype.

In conclusion, *Salmonella* shedding in naturally infected pigs was found to peak in the early grower stage and decline in the mid-finishing stage. From the peak of *Salmonella* infection in pigs, antibody response was delayed 4 wk with the peak level of antibodies occurring in mid-finishing stage. At slaughter, *Salmonella* was recovered from pigs that were *Salmonella* negative based on fecal culture for 7 to 8 wk prior. The absence of *Salmonella* detection in fecal samples in pigs in the late finisher stage is not an indication that *Salmonella* will not be found in tissue at the time of slaughter. Future studies applying serological testing to identify asymptomatic *Salmonella*-carrier pigs is important. Furthermore, the presence of multiple serotypes in pigs may result in reinfections and lack of cross-protection among some *Salmonella* serogroups. Further research assessing *Salmonella* serotypes can be helpful in understanding which serotypes cause prolonged *Salmonella* shedding and are more likely to colonize in tissue.
Acknowledgments

We acknowledge the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) — University of Guelph Research Partnership, OMAFRA Food Safety Research Program, Swine Innovation Porc, and Huvepharma for financial support.

References

Evaluation of the efficacy of a novel porcine circovirus type 2 synthetic peptide vaccine

Jiwoon Jeong, Changhoon Park, Seeun Kim, Su-Jin Park, Ikkae Kang, Kee Hwan Park, Chanhee Chae

Abstract

A novel porcine circovirus type 2 (PCV2) peptide vaccine comprised of a consensus capsid (Cap) protein domain encoded by open reading frame 2 was developed to control PCV2 infection. The efficacy of the vaccine was evaluated against a commercial baculovirus-expressed recombinant PCV2 subunit vaccine based on the Cap protein. The amino acid sequence of this Cap protein was designed based on the alignment of amino acid sequences from different isolates from Europe, North America, and Asia. The vaccine was evaluated in either phosphate-buffered saline or adjuvanted with aluminum hydroxide, cobalt oxide, or liposome. Overall the PCV2 peptide vaccine was less efficacious against PCV2 challenge compared with the commercial PCV2 vaccine. The peptide vaccine was the most efficacious when liposome was used as an adjuvant, significantly (P < 0.05) reducing viremia while increasing the levels of neutralizing antibodies and interferon-γ secreting cells. This suggests, in the presence of liposome, the peptide vaccine was able to elicit both humoral and cellular immune responses.

Résumé

Un nouveau vaccin peptidique contre le circovirus porcin de type 2 (CVP2) contenant un domaine d’une protéine consensus de la capside (Cap) codé par le cadre de lecture ouvert 2 fut développé pour limiter l’infection par CVP2. L’efficacité du vaccin fut évaluée versus celle d’un vaccin commercial CVP2 sous-unitaire recombinant s’exprimant dans un baculovirus et basé sur la protéine Cap. La séquence en acides aminés de cette protéine Cap a été élaborée basée sur l’alignement des séquences d’acides aminés provenant de différents isolats d’Europe, d’Amérique du Nord, et d’Asie. Le vaccin a été évalué dans soit de la saline tamponnée avec du phosphate ou dans un adjuvant contenant de l’hydroxyde d’aluminium, d’oxyde de cobalt, ou des liposomes. Globalement, le vaccin peptidique CVP2 était moins efficace contre une infection défi par CVP2 comparativement au vaccin commercial. Le vaccin peptidique était le plus efficace lorsque les liposomes étaient utilisés comme adjuvant, réduisant de manière significative (P < 0.05) la virémie tout en augmentant la quantité d’anticorps neutralisants et de cellules sécrétant de l’interféron γ. Ceci suggère qu’en présence de liposomes le vaccin lipidique était en mesure d’induire des réponses immunitaires humorale et cellulaire.

(Traduit par Docteur Serge Messier)

Introduction

Porcine circovirus type 2 (PCV2) is considered one of the most important viral pathogens for the swine industry, especially in Asian countries because it is associated with several syndromes and diseases that have been collectively named porcine circovirus associated diseases (PCVAD) (1). Porcine circovirus type 2 has 2 major viral genes, open reading frame (ORF) 1 and ORF2, which together represent 93% of the genome (2). The ORF1 encodes the replication-associated protein and ORF2 encodes the structural capsid (Cap) protein (2).

Currently, vaccination against PCV2 is the major PCVAD control strategy with the first commercial vaccine introduced in 2006 (3). The first commercial PCV2 vaccine was created based on the classical approach of using an inactivated field strain combined with an adjuvant (3). Subsequently, subunit PCV2 and chimeric PCV1-2 vaccines have also been introduced into the international market (3). In addition to commercial PCV2 vaccines, different prototype PCV2 vaccines have also been studied extensively during the past 10 y. Such prototypes have included DNA vaccines (4–8) and recombinant subunit vaccines expressing PCV2 viral proteins (4,9–14). Even though a variety of prototype PCV2 vaccines have been developed over the last decade, none have shown an efficacy similar to the commercial PCV2 vaccines that are currently in use.

A peptide vaccine is likely the next generation candidate to replace the current commercial PCV2 subunit vaccines based on the Cap protein. Synthetic peptides are 10 to 30 amino acids long and are designed to represent the specific immune-epitope of the antigen (15). The advantage of the short immunogenic peptides is that they can induce a protective immunity without allergic and detrimental immune responses. Another advantage is the ease of production and the reduced manufacturing cost compared to conventional vaccines (16). Furthermore, the specific peptide can be selected and modified with bioinformatics tools to increase its immunogenicity (17). Therefore, peptide vaccines have a bright future as potential prophylaxis for a variety of diseases (18). On the other hand, the
current downside of peptide vaccines is the limited immunogenicity due to the relatively small size of the antigen (16). To solve this problem and to efficiently elicit a protective immune response, peptide vaccines have to be improved by using potent immunostimulatory adjuvants. To date no peptide vaccines against PCV2 have been developed. The objective of the current study was to develop a PCV2 peptide vaccine and evaluate its efficacy in combination with different adjuvants. The efficacy was compared with a current commercial PCV2 subunit vaccine.

### Materials and methods

**Consensus capsid protein-based synthetic peptide vaccine**

Amino acid sequences of Cap proteins of 94 PCV2 isolates were obtained from the National Center for Biotechnology Information protein database. A consensus sequence for Cap proteins encoded by ORF2 was obtained by aligning amino acid sequences from different isolates using computer software (Clustal Omega software; EMBL-EBI, Cambridgeshire, United Kingdom) based on the Clustal W algorithm (19). Five consensus amino acid sequences were designed: NH\(_2\)-TRLSRFTGYTIKRTTVKCOOH (peptide number 47–63), NH\(_2\)-YHSRYFT-COOH (peptide number 156–162), NH\(_2\)-PVLDSTIDYFQPNNKNRNLWLCOOH (peptide number 165–185), NH\(_2\)-HVGLGTAFENSITYQDYN-COOH (peptide number 195–212), and NH\(_2\)-TMYVQFREFNLDPLL-N-COOH (peptide number 216–233).

The 5 synthetic peptides were synthesized using solid-phase peptide synthesis and purified by high performance liquid chromatography (COSMO Gene Tech, Seoul, Republic of Korea). The purity of the peptides was > 95%. To prepare PCV2 peptide solution, 2 mg of each synthetic peptide was dissolved in 1 mL of 2 × PBS and then 1 mL from each peptide solution was mixed together.

**Preparation of the peptide vaccine dissolved in phosphate-buffered saline**

Equal volumes of PBS (10 mM, pH 7.4) and PCV2 peptide solution (2 mg/mL in 2 × PBS) were mixed on a magnetic plate stirring at 45 ◦C for 24 h. The vaccine dissolved in PBS was administered into pigs within 4 h of formulation.

**Preparation of peptide vaccine adjuvanted with aluminum hydroxide gel**

Equal volumes of aluminum hydroxide gel (Rehydragel LV; General Chemical, Berkeley Heights, New Jersey, USA) and PCV2 peptide solution (2 mg/mL in 2 × PBS) were mixed on a magnetic plate stirring at 45 ◦C for 24 h. The resulting aluminum hydroxide gel-formulated PCV2 peptide vaccine was used to immunize pigs within 4 h of formulation.

**Preparation of peptide vaccine adjuvanted with cobalt oxide**

The dispersion was done with a slight modification to previously described methods (20,21). Briefly, cobalt oxide (Co\(_3\)O\(_4\)) nanoparticles in deionized water (500 µg/mL) were sonicated using a probe sonicator (220–240 V, 10.3A; Philip Harris Scientific, Lichfield, United Kingdom) at 40% power for 1 min to break up aggregation. Then, equal volumes of the PCV2 peptide solution (2 mg/mL in 2 × PBS) were added and mixed by vortex for 5 min. The hydrodynamic size and zeta potential were analyzed (Zetasizer, Nano-ZS90; Malvern, Malvern Hills, United Kingdom). Finally, a 1-mL dose containing 500 µL of PCV2 peptide (2 mg/mL in PBS) and 500 µL of Co\(_3\)O\(_4\) nanoparticle adjuvant (500 µg/mL) was prepared and stored at 4°C until use.

**Preparation of peptide vaccine adjuvanted with liposome**

For the preparation of liposome nanoparticles, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE; Avanti Polar Lipids, Alabaster, Alabama, USA), dihexadecyl phosphate (DCP; Sigma-Aldrich, St. Louis, Missouri, USA), and cholesterol (Chol; Avanti Polar Lipids) were mixed at a ratio of 3.18:0.55:1.55 (w/w/w) according to techniques previously described (22). Briefly, lipid films were created in a glass vial by evaporating the organic solvent [mixture of chloroform and methanol at a ratio of 6.5:3.5(v/v)] under a steady stream of nitrogen gas. Traces of organic solvent were removed by keeping the films in a vacuum desiccator overnight. For the preparation of the liposome-peptide formulation, lipid films were hydrated for 12 h by adding PCV2 peptide solution (2 mg/mL in 2 × PBS). The suspensions were then sonicated in a bath-type sonicator (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) for 10 min followed by extrusion (Hamilton Company, Reno, Nevada, USA) through 400, 200, and 100 nm membrane filters and were stored at 4°C before use. The hydrodynamic size and zeta potential were measured following the manufacturer’s instructions (Zetasizer, Nano-ZS90; Malvern).

**Experimental design**

A total of 49 colostrum-fed, cross-bred, conventional piglets were purchased at 5 d of age from a commercial farm. All piglets were seronegative for PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and Mycoplasma hyopneumoniae and were non-viremic for PCV2 and PRRSV when tested by real-time polymerase chain reaction (RT-PCR). Their sows were also seronegative and non-viremic for PCV2.

This study used a randomized, blinded, controlled design. Pigs were randomly divided into 7 groups (7 pigs per group) using the random number generation function (Excel; Microsoft Corporation, Redmond, Washington, USA; Table I). The PCV2 peptide vaccine dissolved in PBS (VacPBS/Ch), PCV2 peptide vaccine adjuvanted with aluminum hydroxide (VacAH/Ch), PCV2 peptide vaccine adjuvanted with cobalt oxide (VacCO/Ch), and PCV2 peptide vaccine adjuvanted with liposome (VacLP/Ch) was administered intramuscularly as two 1.0 mL doses at 7 and 21 d of age in the right side of the neck. A dosage of 1 mg of peptide in each vaccine was administered into pigs. Pigs in VacCirco/Ch were injected with a commercial PCV2 subunit vaccine (CircO-FLEX; Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA) at 21 d of age according to the manufacturer’s recommendations. Pigs in UnVac/Ch and UnVac/UnCh were administered an equal volume of PBS (0.01M, pH 7.4, 2.0 mL) at 7 and 21 d of age in the same anatomic location.
At 49 d of age, pigs in VacPBS/Ch, VacAH/Ch, VacCO/Ch, VacLP/Ch, VacCirco/Ch, and UnVac/Ch were inoculated intranasally with 2 mL of PCV2b [strain SNUVR000463; 5th passage; 1.0 × 10^5 tissue culture infectious dose 50%, (TCID_{50}/mL)]. The pigs in UnVac/UnCh were inoculated intranasally with 2 mL of PBS.

All pigs were comingled and randomly assigned to 1 of 2 rooms. At the time of challenge, the negative control (unvaccinated and unchallenged) group was moved separately into a similar room. Blood samples were collected at −42 (7 d of age), −28 (21 d of age), 0 (49 d of age), 7, 14, 21, and 28 (77 d of age) d post-challenge (dpc). Superficial inguinal lymph nodes were collected for histopathology and immunohistochemistry. All of the methods were approved by the Seoul National University Institutional Animal Care and Use Committee prior to the study.

### Quantification of PCV2 DNA in blood

DNA was extracted from serum samples using a kit (QIAamp DNA mini kit; Qiagen, Crawley, United Kingdom) and was used to quantify PCV2 genomic DNA copy numbers by RT-PCR as previously described (23). To construct a standard curve, RT-PCR was done in quadruplicate in 10-fold serial dilution of PCV2b and PCV2b recombinant plasmid as previously described (23).

### Serology

The serum samples were tested using a serum virus neutralization (SVN) test (24). Neutralizing antibody (NA) titers were expressed as the reciprocal of the highest serum dilution in which no reduction or higher than 80% reduction of viral replication was detected in the PK15 cell compared with the virus control. Serum samples were considered to be positive for NA titers if the titer was greater than 2.0 (log_{2}).

### Enzyme-linked immunosspot (ELISPOT) assay

The numbers of PCV2-specific interferon-γ secreting cells (IFN-γ-SC) were determined in peripheral blood mononuclear cells (PBMC) as previously described (25).

### Histopathology

Tissue sections of lymph nodes were blindly examined by 2 different veterinary pathologists (Jeong and Chae). For the morphometric analysis of histopathological lesion scores in lymph nodes, 3 superficial inguinal lymph node sections were examined “blindly” as previously described (26). The lymphoid lesion scores ranged from 0–3; 0 — no lymphoid depletion or granulomatous replacement; 1 — mild lymphoid depletion; 2 — moderate lymphoid depletion; and 3 — severe lymphoid depletion and granulomatous replacement.

### Immunohistochemistry

Thin sections (4 μm) were collected from each sample lymph node and each section was mounted on glass slides that were positively charged (Superfrost/Plus slides; Erie Scientific Company, Portsmouth, New Hampshire, USA), and stored at room temperature. Sections were dewaxed in xylene and rehydrated in PBS (pH 7.4, 0.01 M) for 5 min. The sections were de-proteinized with 0.2 N HCl for 20 min at room temperature. Tissues were then digested at 37°C for 20 min with a proteinase K solution (100 mg/mL in PBS). Slides were rinsed twice with PBS and endogenous alkaline phosphatase was quenched with 20% glacial acetic acid solution for 2 min at 4°C. All slides were then incubated with normal goat serum in PBS (0.1 M, pH 7.4) for 30 min at room temperature to reduce nonspecific binding. Slides were then incubated in primary antibody (rabbit polyclonal anti-PCV2 antibody; Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa, USA) for 1 h at room temperature. Primary antibody was diluted 1:500 in PBS (0.01 M, pH 7.4) containing 0.1% Tween 20. Slides were washed 3 times with Tween 20, and incubated in secondary antibody (biotinylated goat anti-rabbit IgG (Dako, Glostrup, Denmark) for 1 h at 36°C. Secondary antibody was diluted 1:200 in Tween 20. The slides were again washed with Tween 20 and incubated with streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals, Mannheim, Germany) for 1 h at 36°C. The sections were equilibrated with Trisbuffer (pH 8.2) for 5 min at room temperature and immersed in a solution of red substrate (Boehringer Mannheim, Indianapolis, Indiana, USA) for 10 min at room temperature. The sections were lightly counterstained with Mayer’s hematoxylin.

Positive signal was quantified using the NIH Image J 1.45s Program (http://image.nih.gov/ij/download.html). For each slide of lymph node tissue, 10 fields were randomly selected, and the number of positive cells per unit area (0.25 mm^2) was counted. The mean values were also calculated (27).

### Table I. Lymphoid lesion score and porcine circovirus type 2 (PCV2)-antigen score in the different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>PCV2 peptide vaccine with adjuvant</th>
<th>PCV2 challenge</th>
<th>Lymphoid lesion score</th>
<th>PCV2-antigen score</th>
</tr>
</thead>
<tbody>
<tr>
<td>VacPBS/Ch</td>
<td>Peptide vaccine-PBS</td>
<td>Yes</td>
<td>1.14 ± 0.38^ab</td>
<td>9.43 ± 1.72^a</td>
</tr>
<tr>
<td>VacAH/Ch</td>
<td>Peptide vaccine-Al(OH)_3</td>
<td>Yes</td>
<td>0.71 ± 0.76^abc</td>
<td>8.14 ± 1.35^a</td>
</tr>
<tr>
<td>VacCO/Ch</td>
<td>Peptide vaccine-Co_3O_4</td>
<td>Yes</td>
<td>0.71 ± 0.49^c</td>
<td>7.71 ± 1.80^a</td>
</tr>
<tr>
<td>VacLP/Ch</td>
<td>Peptide vaccine-liposome</td>
<td>Yes</td>
<td>0.43 ± 0.53^cd</td>
<td>7.14 ± 1.95^c</td>
</tr>
<tr>
<td>VacCirco/Ch</td>
<td>Commercial vaccine</td>
<td>Yes</td>
<td>0.29 ± 0.49^d</td>
<td>4.86 ± 0.80^b</td>
</tr>
<tr>
<td>UnVac/Ch</td>
<td>No</td>
<td>Yes</td>
<td>1.57 ± 0.53^e</td>
<td>18.43 ± 5.50^e</td>
</tr>
<tr>
<td>UnVac/UnCh</td>
<td>No</td>
<td>No</td>
<td>0.14 ± 0.38^f</td>
<td>0^d</td>
</tr>
</tbody>
</table>

^a,b,c,d Statistically significant differences (P < 0.05) among groups.
Statistical analysis

Prior to statistical analysis, all RT-PCR and NA data were transformed to log_{10} and log_{2} values, respectively. Continuous data (PCV2 DNA, PCV2 serology, PCV2-specific IFN-\(\gamma\)-SC) were analyzed using a repeated measures analysis of variance (ANOVA). Discrete data (lymphoid lesion score and PCV2 antigen score) were analyzed by using Chi-square and Fisher’s exact tests. A value of \(P < 0.05\) was considered to be significant.

Results

Sensitivity of PCV2 primers and probe

The coefficients of determination (\(R^2\)) were over 0.99 for all the regressions of the standard curves. By using the cycle threshold (Ct) values and the regression equations of standard curves, the DNA copy number detected was calculated for RT-PCR reaction with a detection limit estimated to be 25 DNA viral genome copies/RT-PCR reaction. The Ct value obtained at 25 DNA viral genome copies/RT-PCR reaction was 40. All results with a Ct value \(\leq 40\) were considered to be negative for PCV2.

Quantification of PCV2 DNA in blood

Porcine circovirus 2 DNA was not detected in any of the serum samples from any of the 7 groups from -42 to 0 dpc. In the inter-group comparisons, pigs from the VacAH/Ch, VacCO/Ch, VacLP/Ch, and VacCirco/Ch groups had significantly \((P < 0.05)\) less PCV2 genomic copies in their sera compared to pigs from the UnVac/Ch at 7, 14, 21, and 28 dpc. Pigs from the VacPBS/Ch group had significantly \((P < 0.05)\) less PCV2 genomic copies compared when compared to pigs from the VacPBS/Ch, VacAH/Ch, VacCO/Ch, and UnVac/Ch groups at 14 and 21 dpc. Pigs from the VacAH/Ch, VacCO/Ch, and VacLP/Ch groups had significantly \((P < 0.05)\) less PCV2 genomic copies compared with pigs from the VacPBS/Ch and UnVac/Ch groups at 14 and 21 dpc. No genomic copies of PCV2 were detected in the serum of pigs from the UnVac/UnCh group throughout the experiment (Figure 1).

Neutralizing antibodies titters

There was a significant difference \((P < 0.05)\) in the NA titers among the groups. In the inter-group comparisons, pigs from the VacCirco/Ch group had significantly \((P < 0.05)\) higher NA titers compared to pigs from the VacPBS/Ch, VacAH/Ch, VacCO/Ch, and UnVac/Ch groups at 0, 7, 14, 21, and 28 dpc. Pigs from the VacLP/Ch group had significantly \((P < 0.05)\) higher NA titers compared to pigs from the VacPBS/Ch, VacAH/Ch, VacCO/Ch, and UnVac/Ch groups at 0 and 14 dpc. Pigs from the VacAH/Ch group had significantly \((P < 0.05)\) higher NA titers compared to pigs from the VacPBS/Ch, VacCO/Ch, and UnVac/Ch groups at 7, 14, 21, and 28 dpc. No NA was detected in pigs from the UnVac/UnCh group throughout the experiment (Figure 2A).

Porcine circovirus type 2-specific interferon-\(\gamma\) secreting cells

In the inter-group comparisons, pigs from the VacCirco/Ch group had significantly \((P < 0.05)\) higher numbers of PCV2-specific IFN-\(\gamma\)-SC compared to pigs from the VacPBS/Ch, VacAH/Ch, VacCO/Ch, VacLP/Ch, and UnVac/Ch groups at 0, 7, 14, and 21 dpc. Pigs from the VacLP/Ch group had significantly \((P < 0.05)\) higher numbers of PCV2-specific IFN-\(\gamma\)-SC compared to pigs from the VacPBS/Ch, VacAH/Ch, VacCO/Ch, and UnVac/Ch groups...
at -28, 0, 7, 14, 21, and 28 dpc. Pigs from the VacCO/Ch group had significantly (P < 0.05) higher numbers of PCV2-specific IFN-γ-SC compared to pigs from the VacPBS/Ch group at 7 and 14 dpc. No PCV2-specific IFN-γ-SC was detected in pigs from the UnVac/UnCh group throughout the experiment (Figure 2B).

**Histopathology and immunohistochemistry**

The lymphoid lesion scores were significantly (P < 0.05) lower in pigs from the VacCirco/Ch group compared to pigs from the VacPBS/Ch, and UnVac/Ch groups. Pigs from the VacCO/Ch and VacLP/Ch groups had significantly (P < 0.05) lower lymphoid lesion scores compared to pigs from the UnVac/Ch groups. The PCV2-antigen score was significantly (P < 0.05) lower in pigs from the VacCirco/Ch group compared to pigs from the VacPBS/Ch, VacAH/Ch, VacCO/Ch, VacLP/Ch, and UnVac/Ch groups. Pigs from the VacPBS/Ch, VacAH/Ch, VacCO/Ch, and VacLP/Ch groups (Figure 3A) had significantly (P < 0.05) lower PCV2-antigen scores compared to pigs from the UnVac/Ch group (Figure 3B). No lymphoid lesions and PCV2-antigen was detected in any of the lymph nodes of pigs from the UnVac/UnCh group (Table I).

**Discussion**

The results presented in this study demonstrate that peptide vaccines, only when using liposome as an adjuvant, are able to elicit both humoral (i.e., NA) and cellular (i.e., IFN-γ-SC) immune responses in pigs in contrast with 2 other adjuvants, such as aluminum hydroxide and cobalt oxide. Aluminum hydroxide is able to induce humoral immunity by preferentially priming Th2-type immune responses (28). This may explain why a PCV2 peptide vaccine adjuvanted with aluminum hydroxide induces a good humoral but poor cellular immune response. Cobalt oxide activates Th1-type immune responses (20), which may explain the predominant induction of cellular immune responses by a PCV2 peptide vaccine.
adjuvanted with cobalt oxide. In contrast, the PCV2 peptide vaccine using liposome as an adjuvant induced strong humoral as well as cellular immune response. Liposomes are well known for their ability to enhance the exposure of antigen and immunostimulators to the antigen presenting cells (16,29,30). They act as a vehicle or delivery system of antigen to the antigen presenting cell, thereby enhancing both humoral and cellular immune response more effectively than the rest of the adjuvants used in this study.

Selecting the proper immunogenic domain is critical for the efficacy of a peptide vaccine. The synthetic peptide vaccine developed for this study is based on critical immunogenic epitopes located in the Cap protein and is designed to induce PCV2-specific neutralizing antibodies. The Cap protein is the major structural protein of PCV2 (31) and immunogenicity analyses of the Rep (encoded by ORF1) and Cap (encoded by ORF2) proteins, determined that Cap is the most important immunogenic domain and the main neutralizing epitope whereas the Rep protein exhibited only weak immunogenicity (4). Four linear immunodominant regions and at least 5 overlapping conformational epitopes within residues 47–85, 165–200, and 230–233 (32) of the PCV2 Cap, were identified by a PEPSCAN analysis (33). Furthermore, 5 linear B-cell epitopes were finely defined with synthetic peptides, and the critical residues in epitope 231–233 and 156–162 were identified as Proline and Tyrosine, respectively (34). Moreover, a PCV2 peptide vaccine adjuvanted with liposome exhibits cross-protection against other genotypes, such as PCV2a and PCV2d (personal observation by Dr. Chae). These results suggest that the synthetic peptides designed in this study are common immunogenic epitopes resulting in broad cross-protection. In this study, only one PCV2b strain was used as a challenge and further studies are needed to test different PCV2b isolates from different countries and/or different regions within the same countries.

Induction of humoral and cellular immune responses by the PCV2 synthetic peptide vaccine are clinically significant because these immune responses are critical in reducing PCV2 viremia. Porcine circovirus 2 in the blood is a major contributor in the development of PCVAD. The PCV2 viremia facilitates viral distribution throughout the lymphoid tissues (35) and high titers of PCV2 load in blood are associated with the development of PCVAD (36,37). A positive correlation was observed between the induction of protective immunity and the reduction of PCV2 viremia and clinical PCVAD (37–39). The peptide vaccine seems to elicit higher levels of protective immunity, including humoral (i.e., NA) and cellular (i.e., IFN-γ-SC), when formulated with liposome as an adjuvant, effectively reducing PCV2 viremia and controlling PCV2 infection.

Despite the fact that the PCV2 peptide vaccine designed in this study was capable of inducing humoral and cellular immune responses, it was less efficacious than the commercial PCV2 subunit vaccine that is based on the whole Cap protein. One reason for this difference could be that the peptides in our candidate vaccine might not fold into an identical structure(s) to that same region in the full Cap protein. Nevertheless, a mixture of peptides consisting of 7, 17, 17, and 21 amino acids from the sequence of the Cap protein can be recognized by antigen presenting cells and are able to elicit immune responses similar to the full length Cap protein. Further studies are needed to determine how the synthetic peptides interact with antigen presenting cells compared to the full length Cap protein.

For a peptide vaccine to be effective, an accurate immunogenic epitope should be identified and an effective adjuvant be selected. In the present study, the PCV2 peptide vaccine using immunogenic epitopes selected from the Cap protein combined with liposome as an adjuvant elicits strong protective immune responses and can control PCV2 infection effectively. The immune response and protection was similar to a PCV2 DNA vaccine used in a previous study (40). Peptide vaccines are more promising because they have considerable advantages compared to DNA vaccines. Current advancement in bioinformatics, proteomics, immunogenomics, and structural biology have ignited a new field of vaccinomics where computer assisted approaches are used to identify suitable peptide targets for eventual development of efficient peptide vaccines (17). However despite the many advantages, peptide vaccines do have certain drawbacks. One drawback is that not enough protective immunity is elicited after administration of a single dose compared with one dose of a conventional commercial vaccine. Two-dose administration is needed to elicit similar protection to one dose of a conventional commercial vaccine. Therefore, it is necessary to develop a more effective adjuvant or delivery system that elicits a sufficient

![Figure 3. Results of immunohistochemistry for the detection of porcine circovirus type 2 (PCV2)-antigen in the lymph nodes of pigs in the VacLP/Ch (A) and UnVac/Ch (B) groups 28 d after challenge.](image)
immune response even with one dose. Further studies are necessary to develop a single dose peptide vaccine that is as efficacious as a single dose commercial conventional PCV2 vaccine.

Acknowledgments

This research was supported by contract research funds of the Research Institute for Veterinary Science from the College of Veterinary Medicine and by the BK 21 Plus Program (Grant no. 5360-20150100) for Creative Veterinary Science Research.

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Effect of a commercially available fish-based dog food enriched with nutraceuticals on hip and elbow dysplasia in growing Labrador retrievers

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Abstract

The aim of this study was to evaluate the prevalence of hip and elbow dysplasia in a group of growing Labrador retrievers fed a fish-based diet enriched with nutraceuticals with chondroprotective properties. The puppies ranged from 3 to 12 mo of age and were divided into 2 groups, each fed a different diet. The control diet consisted of a high quality, chicken-based dog food, while the test diet was a fish-based dog food, enriched with nutraceuticals. Hip and elbow joints were radiographed and scored at 6 and 12 mo of age. Overall, 42 dogs completed the study. At 12 mo of age, no differences were found between the groups in the prevalence of hip and elbow dysplasia, although dogs fed the fish-based food enriched with nutraceuticals had a less severe grade of osteoarthritis at 12 mo. It was concluded that the fish-based diet with nutraceuticals did have beneficial effects on the development of severe osteoarthritis.

Résumé

L’objectif de la présente étude était d’évaluer la prévalence de dysplasie de la hanche et du coude dans un groupe de chiens Labrador en croissance nourris avec une diète à base de poisson enrichie de nutraceutiques ayant des propriétés chondroprotectrices. L’âge des chiots variait de 3 à 12 mois et ils ont été divisés en deux groupes, chacun étant nourri avec une diète différente. La diète témoin consistait d’un aliment de haute qualité pour chien à base de poulet, alors que la diète test était un aliment pour chien à base de poisson et enrichi avec des nutraceutiques. Les articulations des hanches et des coudes ont été radiographiées à 6 et 12 mois d’âge. Un total de 42 chiens a complété l’étude. À 12 mois d’âge, aucune différence n’a été trouvée entre les groupes dans la prévalence de dysplasie de la hanche et du coude, bien que les chiens nourris avec la diète à base poisson enrichie de nutraceutiques avaient un score d’ostéarthrite moins sévère à 12 mois. Il a été conclu que la diète à base de poisson enrichie de nutraceutiques avait des effets bénéfiques sur le développement d’ostéarthrite sévère.

(Traduit par Docteur Serge Messier)
Table I. Assessments of radiographic analyses of elbow joints.

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elbow osteophyte</td>
<td>0</td>
<td>No osteophytes</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Largest osteophytes &lt; 2 mm</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Largest osteophytes 2 to 5 mm</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Largest osteophytes &gt; 5 mm</td>
</tr>
<tr>
<td>Medial coronoid disease</td>
<td>0</td>
<td>Normal coronoid</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild change in coronoid shape</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Marked change in coronoid shape</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Fragmented coronoid</td>
</tr>
<tr>
<td>Ulnar subtrochlear</td>
<td>0</td>
<td>No sclerosis</td>
</tr>
<tr>
<td>sclerosis</td>
<td>1</td>
<td>Mild sclerosis, trabecular pattern</td>
</tr>
<tr>
<td></td>
<td></td>
<td>still seen</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate sclerosis, trabecular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pattern slightly unclear</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe sclerosis, trabecular pattern</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cannot be seen</td>
</tr>
<tr>
<td>Humeral radioulnar</td>
<td>0</td>
<td>No incongruity</td>
</tr>
<tr>
<td>incongruity</td>
<td>1</td>
<td>Mild incongruity &lt; 2 mm</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate incongruity 2 to 3 mm</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe incongruity &gt; 3 mm</td>
</tr>
</tbody>
</table>

incongruity; distraction index (DI) (6 mo); and Norberg angle (12 mo). The secondary outcome was measured at 2 endpoints (6 and 12 mo).

Based on a published study on hip dysplasia (12), a sample size of 17 dogs/group was calculated in order to meet the minimum requirement for providing 80% confidence in difference detection at a significance level of 0.05. It was determined that 25 dogs/group was an appropriate initial sample size, which accounted for a dropout rate of 10%. All veterinarians followed guidelines established for good clinical practice and owners provided informed consent for participation.

Either the sire or dam of each litter had previously scored with mild elbow or hip dysplasia. Inclusion criteria for the study were: i) birth in the spring/summer season with the pre-weaning period spent on a farm; ii) participation in off-leash exercise on soft ground; and iii) consumption of the same starter food (processed chicken meal, ground rice, fish meal, with added vitamins, 0.5% colostrum, and 0.047% glucosamine). Exclusion criteria included the presence of acute traumatic injuries or systemic diseases, poor owner compliance, adverse food reactions, and food palatability. Dogs that underwent joint surgery or were lost to followup were also excluded.

At birth, litters of puppies were assigned collars of different colors. At 2 mo, colors were changed to labels from a number sequence. Dogs were distributed equally into 2 gender-balanced groups and were then randomly allocated into the 2 test groupings using a computer-generated randomization list (GraphPad Prism 7; GraphPad Software, San Diego, California, USA) by one author (FDI) and the breeder. At 2 mo, all puppies underwent a complete physical and orthopedic examination by private practice vets. The results of both hip and elbow orthopedic examinations were used as a baseline. A dropout rate of 10%.

Table II. Assessments of radiographic analyses of hip joints.

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Femoral head does not fit in a circle due</td>
</tr>
<tr>
<td></td>
<td></td>
<td>to bone loss</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Obvious bone loss and distinct exostosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>giving a slight conical appearance</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Very gross remodelling with marked bone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>loss and much new bone</td>
</tr>
<tr>
<td>Position of femoral</td>
<td>0</td>
<td>Femoral head is well centered in the</td>
</tr>
<tr>
<td>head</td>
<td></td>
<td>acetabulum or lies medially to the DAE</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Center of femoral head is superimposed on</td>
</tr>
<tr>
<td></td>
<td></td>
<td>the DAE</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Center of femoral head is just lateral to</td>
</tr>
<tr>
<td></td>
<td></td>
<td>the DAE</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Center of femoral head is well</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lateral to, and just touches, the DAE</td>
</tr>
<tr>
<td>Cranial acetabular</td>
<td>0</td>
<td>Sharp, clean-cut junction of the DAE and</td>
</tr>
<tr>
<td>rim</td>
<td></td>
<td>CRAE</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Very small exostosis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Small exostosis or very small facet</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Gross exostosis and/or facet and/or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>moderate bilabiation</td>
</tr>
<tr>
<td>Femoral head and</td>
<td>0</td>
<td>Smooth rounded profile</td>
</tr>
<tr>
<td>neck exostosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slight exostosis in ring form and/or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dense vertical line adjacent to trochanter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ic fossa (Morgan line)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Distinct exostosis in ring form</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Massive exostosis giving a mushroom-like</td>
</tr>
<tr>
<td>Norberg angle</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>$\geq 105^\circ$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$100^\circ$ to $&lt; 105^\circ$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$95^\circ$ to $&lt; 100^\circ$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$&lt; 95^\circ$</td>
</tr>
</tbody>
</table>

DAE — dorsal acetabular edge; CRAE — cranial acetabular edge.

Dogs in the control group were fed a high quality food for puppies, while dogs in the treatment group were fed a specific fish-based food for puppies, supplemented with nutraceuticals. These feeding regimes were maintained until the dogs reached 12 mo of age. Both foods were commercially available kibble. The control and the nutraceutical-supplemented diets were carefully adjusted to provide similar calorific intake and to satisfy the nutritional requirements of growing dogs and puppies. The analytical composition of the diets was: crude protein 26%; carbohydrates 34.2%; crude oils and fats 13% (omega 3, 2.21% and omega 6, 2.18%); crude fiber 2.5%; crude ash 8%; and metabolized energy, 3 464 kcal/kg. The control food contained dehydrated chicken meal as the main protein source. The dietary test food contained fish meal and combined kibble and cold-pressed tablets mixed in the same package, with the tablets making...
up 6% to 7% w/w of the completed food. The tablets consisted of hydrolyzed fish and vegetable protein, supplemented with vegetal glucosamine, chondroitin sulphate, chitosamine, *Boswellia serrata*, devil’s claw (*H. procumbens*), green-lipped mussel (*P. canaliculus*), and omega-3/6 fatty acids in a ratio of 1:1.

Owners were instructed to supply an age-adjusted amount of food 3 times a day and to control the puppy’s activities by avoiding prolonged or intense physical activity, such as walking up stairs or running after a ball or a stick at high speed (2). Owners were telephoned when their puppies reached 4, 8, 10, and 30 mo of age to answer a questionnaire about feeding patterns, physical exercise and housing, difficulties in jumping/walking, or perception of lameness. The interviewer (FM) was unaware of the group assignment at the time of the interviews.

Body weight and body condition (scale from 1 to 5) (6) were monitored throughout the study (at birth and at 4, 6, 8, 10, 12, and 30 mo). Dogs were clinically and radiographically evaluated at 6 and 12 mo. The clinical assessment involved evaluation by an observer (AV), blind to the randomization of dog subjects, who assigned a score ranging from 0 to 3 (none, mild, moderate, severe) for various orthopedic variables (lameness, range of motion, swelling, pain), based on a previous report (1).

Hip and elbow joints were radiographed at 6 and 12 mo of age under sedation. Two trained operators (SM and AV, both with PhDs in Domestic Animal Orthopedics), who were blind to the randomization of dogs, carried out the radiographic examinations. Elbow joints were radiographed in 45° flexed mediolateral and craniocaudal projections (1). Hip joints were radiographed in the standard ventrodorsal hip-extended view. At 6 mo of age, an extra radiograph was done for each hip in ventrodorsal projection with distraction. A trained radiologist (GG) evaluated all the radiographs independently, unaware of which group the dog on the radiograph belonged to. At 6 and 12 mo of age, the distraction index (DI) was calculated. A DI value of < 0.4 was considered normal, > 0.7 was considered abnormal, and values of 0.41 to 0.69 were considered unreliable (15) (Table II). The Norberg angle was calculated (14).

Table III. Prevalence of elbow and hip dysplasia between groups. No statistically significant differences were found.

<table>
<thead>
<tr>
<th>Age</th>
<th>Control group</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elbow dysplasia</td>
<td>Hip dysplasia</td>
</tr>
<tr>
<td>6 mo</td>
<td>23%</td>
<td>38%</td>
</tr>
<tr>
<td>12 mo</td>
<td>69%</td>
<td>38%</td>
</tr>
</tbody>
</table>

Figure 1. Influence of diet on the distribution of osteoarthritis grade of dogs at 12 mo. The worst elbow and hip joint of each dog was considered. Blue indicates the control diet and red indicates the test diet.

The prevalence of hip and elbow dysplasia between the 2 groups was analyzed by a chi-squared test. Binary logistic regression was used to determine any associations between the control group and secondary outcomes (using data composites at 6 and 12 mo), including gender (female versus male) as a dependent variable to control for eventual imbalances between groups. Probabilities with 95% confidence intervals (CIs) were used to quantify the strength of these associations. The Hosmer-Lemeshow statistic was used in order to assess goodness-of-fit of the model. Correlations between composite measures for severity of hip and elbow OA were calculated using the Spearman rank correlation coefficient. Mann-Whitney U-test was used to compare clinical assessment and radiological scores for each single factor previously mentioned, between test and control groups at the 2 endpoints (6 and 12 mo of age). P < 0.05 was considered significant.

Fifty Labrador retriever puppies, 26 males and 24 females (mean age: 60.04 ± 0.13 d standard deviation (SD); mean weight: 7 ± 1 kg), from 6 different litters from the same breeder, were enrolled. Twenty-five dogs were allocated to the control group and 25 to the treatment group. A total of 42 dogs completed the study (22 males and 20 females), 20 dogs in the control group, 22 in the treatment group. Eight dogs were excluded because of poor owner compliance (n = 6) or refusal of the food (n = 2). Overall, 93% of the puppies were kept both indoors and outdoors, with 7% kept outdoors only. All dogs were normal at physical and orthopedic examination at 2 mo. None of the dogs had been neutered or spayed or had undergone major surgery. The birth weight was 375 ± 50 g.

There were no differences in body weight or body condition score between the 2 groups at any time point. Overall, only 3 dogs had clinical signs. No differences were found between the 2 groups at 6 and 12 mo of age for both the pelvic and thoracic limb. No significant differences were found between males and females in the incidence of hip/elbow disease or body weight in either group. There was no significant difference in prevalence of hip and elbow dysplasia in either group, which were the same at 6 and 12 mo. In the univariate analysis, the control diet was associated with a more severe OA at 12 mo of age [odds ratio (OR): 1.17; 95% CI: 1.01 to 1.36; P = 0.038] (Figure 1). The result did not change when adjusted for gender (OR: 1.19; 95% CI: 1.01 to 1.4; P = 0.036). At 6 mo, there was no association between diet group and OA severity in univariate analysis or when adjusting for gender. Severity of elbow dysplasia
was not correlated to severity of hip joint dysplasia at any time point. At 12 mo, dogs in the control group were more severely affected by osteoarthritis in the elbow joint and had a higher grade of elbow joint incongruity than dogs in the test group. At 12 mo, dogs fed the test diet had higher Norberg angle values. No significant difference was found for the other single factors. The telephone questionnaire at 30 mo showed no differences in the presence of lameness than at the assessment at 12 mo.

Dietary supplements can potentially modify some of the underlying processes involved in OA by modulating the inflammatory response, providing nutrients for cartilage repair, and protecting against oxidative damage (7,16). It has been reported that calorie-restricted diets are useful in counteracting the progression of osteoarthritis (17).

No significant differences in the prevalence of hip and elbow OA were identified between the groups in this study. As a secondary outcome, however, severity of OA differed between groups. Dogs fed a fish-based dog food enriched with nutraceuticals had a less severe grade of OA at 12 mo of age. This outcome is in agreement with the results of a recent study, in which oral administration of hyaluronic acid, hydrolyzed collagen, glucosamine, chondroitin sulphate, and gamma oryzanol between 3 to 20 mo significantly decreased the prevalence of clinical signs of elbow OA in a cohort of Labrador retriever dogs (1). Although supplementing with specific chondroprotective properties may have no effects on phenotypic expression of hip and elbow dysplasia, it may have a cumulative protective effect against the progression of radiographic osteoarthritic changes (1). This hypothesis is supported by a recent review of studies on dietary supplements for managing OA in dogs (16).

The use of fish instead of chicken could be another important issue, due to the presence of oxytetracycline residues in poultry bones (18,19). Oxytetracycline residues exert cytotoxic effects, which suggests that the use of poultry bones and deboned meat in pet food is a potential risk (10,18,19). It is possible that the combination of a major supply of omega-3 fatty acids contained in fish oil and fish meal and the cytotoxic effect of oxytetracycline residues in poultry bones could have contributed to the results of the secondary outcome in the present study. However, more studies are needed to test the effects of a single protein source and/or its pollutants on developmental skeletal disease in growing dogs. In this study, no adverse effects could be attributed to the treatment diet, which was also noticeably associated with good product palatability.

The majority of dogs from both groups with radiological diagnoses of OA did not show clinical signs up to 30 mo of age. A combination of strict weight control during growth, associated with moderate exercise, could have played a concomitant role in both groups. Although the results of the secondary outcome may provide some additional information about the effects of a commercially available fish-based dog food enriched with nutraceuticals on the severity of OA, there are some limitations that should be mentioned. Animals were not radiographically followed at 30 mo of age and elbow lesions were not evaluated with computed tomography to detect early lesions, so false negatives were possible (20).

In conclusion, administration of fish proteins and supplementation with nutraceuticals with chondroprotective, anti-inflammatory, and antioxidant properties in the same food did not reduce the prevalence of hip and elbow dysplasia in this sample of dogs. Dogs fed the fish-based food had a less severe grade of OA at 12 mo, however, and therefore this diet did have beneficial effects on the development of severe osteoarthritis.

References


Peak vertical force in a stabilized canine cranial cruciate deficient stifle model: A one-year follow-up

Bertrand Lussier, Alexandre Gagnon, Maxim Moreau, Jean-Pierre Pelletier, Éric Troncy

Abstract

This study aimed to describe the peak vertical force (PVF) over a 1-year period in a stabilized canine cranial cruciate deficient stifle model. Our hypothesis was that PVF would be restored to Baseline (intact) at the end of the follow-up.

Fifteen (> 20 kg) mixed-breed dogs were included in this study. Cranial cruciate ligament was transected on Day (D) 0 followed by lateral suture stabilization at D28. Peak vertical force was acquired at D1, D14, D26, D91, D210 and D357.

When compared to Baseline, the PVF was significantly decreased at D14, D26, and D91. Values at D210 and D357 were not statistically different to Baseline.

This study suggests a return to normal baseline peak vertical force in a canine cranial cruciate deficient stifle model when lateral suture stabilization has been performed 28 days after surgical transection.

Résumé

Le but de cette étude était de rapporter, chez un modèle canin stabilisé suite à une déficience du genou induite par section transversale du ligament croisé crânial (LCC), l’évolution du pic de force vertical (PFV) sur une année. Notre hypothèse était que le PFV serait normalisé à la fin de la période de suivi.

Quinze (>
20 kg) chiens croisés ont été inclus dans l’étude. L’instabilité du genou a été induite par section transversale du ligament croisé crânial au jour (J) 0, suivie par la stabilisation par suture latérale à J28. Le PFV a été acquis à J–1, J14, J26, J91, J210, et J357.

Lorsque comparé à J0, le PFV était significativement diminué à J14, J26 et J91. Les valeurs du PFV à J210 et J357 n’étaient pas différentes qu’à J0.

Cette étude suggère le retour des valeurs normales du pic de force verticale chez un modèle d’instabilité induite du genou lorsque la stabilisation a été réalisée 28 jours après la section transversale du ligament croisé crânial.

This study was approved by the Institutional Animal Care and Use Committee (Rech-1354) in accordance with the guidelines of the Canadian Council on Animal Care. Fifteen dogs (9 males and 6 females) weighing 25.0 ± 2.1 kg [mean ± standard deviation (SD)] were used. Orthopedic and radiographic examinations of the elbows, hips, and stifles were performed to exclude dogs with any pre-existing joint pathology with an emphasis on OA structural changes. Animals were trained to walk on a leash 2 to 3 times per week from day (D) −30 to D −1. The CCLT surgery was performed at D0. Briefly, the cranial cruciate ligament of the right stifle was transected under direct visualization via a limited medial arthrotomy. A positive drawer in flexion and extension confirmed the complete transection. The LSS surgery was performed at D28 as previously described (3). Briefly, the lateral retinaculum was identified and dissected from the lateral joint capsule. The stifle joint was not explored or debrided. A double strand of 130-lb test monofilament nylon (Special mer; Tortue, La Soie Neyme SA, Paris, France) mounted on a needle was passed around the lateral fabella, then through a hole previously created in the tibial tuberosity and passed under the patellar ligament. Both strands were tied independently using square knots. The same

Osteoarthritis (OA) is a highly prevalent musculoskeletal disorder that generates pain and leads to functional disability. Numerous animal models of OA have been developed over the past 50 y. The canine cranial cruciate deficient stifle (CCDS) model, which is created by the transection of the cranial cruciate ligament (CCLT), has a recognized translational value (1). It is an efficient, reproducible, and reliable model of OA secondary to biomechanical and inflammatory insults (2). In an attempt to modulate the onset and severity of OA structural changes, a modified CCDS model was developed. The proposed model consisted in the stabilization of the stifle 28 d after the experimental transection using a lateral suture stabilization (LSS) technique. Gait analysis and more specifically, peak vertical force (PVF) measurements, have been used extensively to describe the functional impairment that prevails in experimentally induced as well as in naturally occurring OA in dogs. To the authors’ knowledge, 1-year functional outcome of the canine CCDS model stabilized by lateral suture has not been evaluated by gait analysis.

The purpose of this study was to describe the PVF evolution over a 1-year period. Our hypothesis was that the PVF would be restored to baseline (intact) over 1 y.

This study was approved by the Institutional Animal Care and Use Committee (Rech-1354) in accordance with the guidelines of the Canadian Council on Animal Care. Fifteen dogs (9 males and 6 females) weighing 25.0 ± 2.1 kg [mean ± standard deviation (SD)] were used. Orthopedic and radiographic examinations of the elbows, hips, and stifles were performed to exclude dogs with any pre-existing joint pathology with an emphasis on OA structural changes. Animals were trained to walk on a leash 2 to 3 times per week from day (D) −30 to D −1. The CCLT surgery was performed at D0. Briefly, the cranial cruciate ligament of the right stifle was transected under direct visualization via a limited medial arthrotomy. A positive drawer in flexion and extension confirmed the complete transection. The LSS surgery was performed at D28 as previously described (3). Briefly, the lateral retinaculum was identified and dissected from the lateral joint capsule. The stifle joint was not explored or debrided. A double strand of 130-lb test monofilament nylon (Special mer; Tortue, La Soie Neyme SA, Paris, France) mounted on a needle was passed around the lateral fabella, then through a hole previously created in the tibial tuberosity and passed under the patellar ligament. Both strands were tied independently using square knots. The same
investigator (B.L.) performed both CCLT and LSS surgeries. A pre-
emptive and multimodal analgesia protocol (i.e., fentanyl patch and
oxydorm or meperidine opioid, as needed in the postoperative
period and peri-articular bupivacaine block) was used. Dogs were
housed in individual runs (1 m × 1.75 m × 2.4 m), each separated
by a panel. After surgical stabilization, they were kept in their cages
for the first 6 wk except for short leash walks 2 to 3 times daily.
Between week 7 and week 16, the duration of the walks gradually
increased to reach 20 min, 3 times daily. Between weeks 17 and 52,
the dogs were given free access to exercise in a large enclosure. They
exercised and socialized in exterior runs (1.35 m width × 9.15 m
length) for a 2-hour period 5 d per week, under the supervision of
an animal care technician. No hands-on rehab was instituted. Peak
vertical force of the right hind limb was recorded at the trot using a
floor mat-based planar force measurement system (Walkway with 4
MatScan sensors 3150; Tekscan, Boston, Massachusetts, USA).
Velocity (1.9 to 2.2 m/s) and acceleration (± 0.5 m/s²) were ensured
using a set of 3 photoelectric cells specially designed (Laboratoire
de communications et d’intégration de la microélectricité, École de
Technologie Supérieure, Montreal, Quebec).

The dogs were walked by the same handler. No or minute tension
was applied on the leash. All passes were unidirectional. Dogs were
observed from behind to make sure that the passes were adequate by
means of assessing: straight head, no body deviation, and no lateral
movement. The first 5 valid trials were obtained for each dog and
then averaged for statistical purposes. Recordings were done at D1,
D14, D26, D91, D210, and D357. A linear mixed model for repeated
measures was used to detect change over time in log-transformed
PVF values (mean of the 5 trials) expressed in kg, using the dog’s
body weight as a covariate. The model included “time” as a fixed
factor and “dog” as a random factor. Analyses were carried using
appropriate software (IBM SPSS Statistics for Windows, version
20.0; IBM, Armonk, New York, USA) with an alpha threshold for
significance set at 5%. Bonferroni correction was applied for multiple
comparisons providing adjusted P-values.

Individual and mean PVF data recorded over time are illustrated
in Figure 1. According to post-hoc analyses, PVF values were signifi-
cantly decreased (*P < 0.05) at D14, D26, and D91. Values at D210 and
D357 were not statistically different from baseline (intact). This
follow-up study reported a return to normal baseline PVF of
the operated limb 1 y after CCLT in dogs having their experimentally
induced CCDS stabilized by LSS. According to PVF, a recognized
objective outcome measure of limb function, a severe impairment
was denoted in CCDS dogs 14 and 26 d following surgery. Then,
following LSS performed 28 d after CCLT, progressive remission
occurred, reaching values that were not statistically different from
baseline (intact) at D210 and D357. These findings indicate that LSS
restored the deficit PVF values to normal baseline PVF in ligament-
transected dogs. Alternatively, as described by the well-established
CCDS model, these values may persist up to 48 mo following sur-
gical transaction (4). However, the complete remission observed in
CCDS-LSS dogs was exclusively based on a biomechanical measure
of the limb function with PVF. The overall clinical outcome may be
quite different as discrepancies were reported between gait analysis
and assessments of chronic pain in dogs with naturally occurring
OA (5).
CCDS. Why then are reported surgical techniques suboptimal when considering return to normal PVF values in spontaneous CCDS? These are 2 different CCDS conditions. We propose that the method of surgical stabilization may not be as important as once thought. The surgeon’s experience and insight into the surgical procedure may also have played a role in the outcome. It may be as important to address the intra-articular degenerative and/or inflammatory processes leading to spontaneous rupture of the CCL. Furthermore, it has been reported that dogs afflicted with chronic CCDS may suffer from nociceptive sensitization leading to alldynia and hyperalgesia (10). We propose this issue also needs to be addressed, particularly with regards to its imbrication with neurogenic inflammation (11).

In conclusion, the procedure described herein was able to restore the operated limb of dogs with experimentally induced CCDS stabilized by LSS, to normal baseline PVF 1 y after CCLT. Why this was observed in an experimental setting and not in dogs with naturally occurring CCDS needs to be further investigated by addressing the biological and neurological aspects of this complex disease. Finally, prudence is necessary when using this model to test pharmacological efficacy, as joint stabilization could prevent or mask further demonstration of a therapeutic effect later in the disease process on PVF. Outcome measures complementary to PVF should be considered to further demonstrate a therapeutic effect. Further studies are required in order to determine a more sensitive functional outcome for long-term follow-up study in this model.

References
