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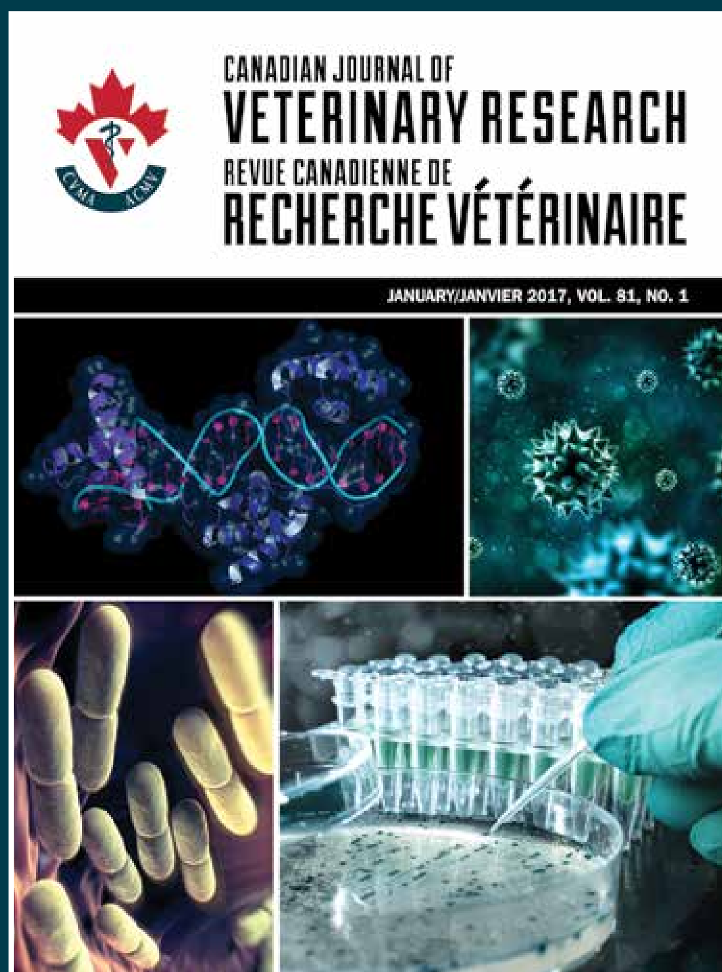

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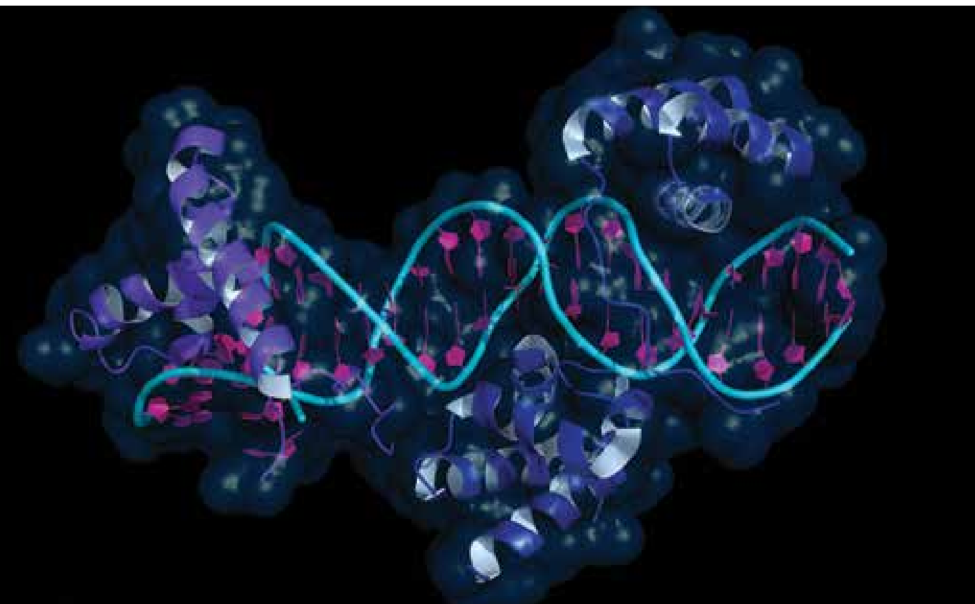

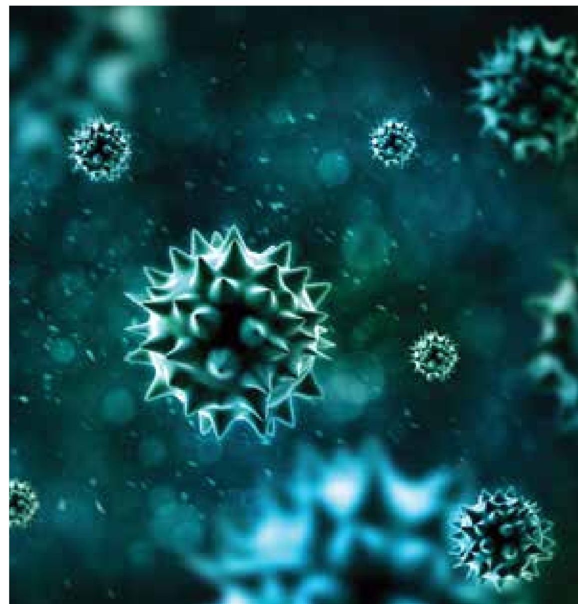

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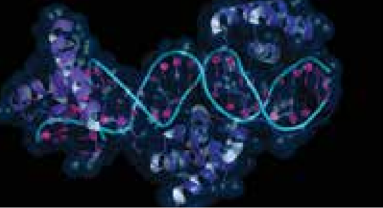

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Induction of humoral immune response in piglets after perinatal or post-weaning immunization against porcine circovirus type-2 or keyhole limpet hemocyanin

Jessica Law, UCVM Class of 2015*, Robert McCorkell, Greg Muench, Katherine Wynne-Edwards, Hermann M. Schaetzl, Cristina Solis, Narges Nourozieh, Regula Waeckerlin, Michael Eschbaumer, Shawn Horsman, Markus Czub

Abstract

The objective of this study was to test the hypothesis that porcine circovirus type-2 (PCV2) vaccination is efficacious when administered in the first week of life. Three groups of pigs were vaccinated with Circumvent either early (at the end of week 1), late (at the end of week 4), or not at all. All 3 groups were later challenged intranasally with PCV2 (at the end of week 5). Two other groups were immunized with keyhole limpet hemocyanin (KLH) as a novel antigen at the end of either week 1 or week 4. Weight, PCV2 genome copy number in serum and saliva, anti-KLH antibody titer, and serum PCV2-neutralizing antibodies were measured weekly. Early PCV2 vaccination or KLH antigen exposure resulted in earlier humoral responses that were slower to develop than in older piglets, yet converged with the responses to later vaccination within 5 wk. Both groups of vaccinated piglets had periods of higher PCV2-neutralizing antibody titers and lower viral levels shortly after weaning and PCV2 challenge, thus supporting the recent labelling of 1 Canadian PCV2 vaccine for use in week 1 and suggesting that early PCV2 vaccination can reduce piglet handling without compromising vaccine efficacy.

Résumé

L'objectif de la présente étude était de vérifier l'hypothèse que la vaccination contre le circovirus porcin de type 2 (CVP2) est efficace lorsqu'administrée durant la première semaine de vie. Trois groupes de porcs ont été vaccinés avec Circumvent soit hâtivement (à la fin de la semaine 1), tardivement (à la fin de la semaine 4), ou pas du tout. Les trois groupes ont plus tard été inoculés par voie intranasale avec CVP2 (à la fin de la semaine 5). Deux autres groupes ont été immunisés avec de l'hémocyanine de patelle (KLH) à titre de nouvel antigène à la fin de soit la semaine 1 ou la semaine 4. Le poids, le nombre de copies du génome de CVP2 dans le sérum et la salive, le titre d'anticorps anti-KLH, et le titre d'anticorps sériques neutralisants CVP2 ont été mesurés à chaque semaine. La vaccination tôt contre CVP2 ou l'exposition à l'antigène KLH a donné des réponses humorales plus hâtives qui étaient plus lentes à se développer que chez les porcs plus vieux, mais qui convergeaient vers les réponses de la vaccination tardive à l'intérieur d'un délai de 5 sem. Les deux groupes de porcelets vaccinés avaient des périodes de titres d'anticorps neutralisants contre CVP2 plus élevés et des charges virales plus basses peu de temps après le sevrage et le challenge avec CVP2, soutenant ainsi l'étiquetage récent d'un vaccin canadien contre CVP2 pour utilisation dans la semaine 1 et suggérant qu'une vaccination tôt contre CVP2 peut réduire la manipulation des porcelets sans compromettre l'efficacité du vaccin.

(Traduit par Docteur Serge Messier)

Introduction

It is suspected that a high proportion of swine herds throughout Canada is seropositive for porcine circovirus type-2 (PCV2), a pathogenic variant of PCV first differentiated from non-pathogenic PCV type-1 (PCV1) in 1998 (1–4). Infection with PCV2 can result in a wide variety of diseases, known as porcine circovirus-associated diseases (PCVAD), including post-weaning multi-systemic wasting syndrome (PMWS), PCV2-systemic disease (PCV2-SD), PCV2-subclinical infection (PCV2-SI), PCV2-reproductive disease (PCV2-RD), and porcine dermatitis and nephropathy syndrome (PDNS) (3). The initiation of intensive vaccination by the Canadian swine industry in 2006 has greatly reduced the incidence of PCVAD and enhanced productivity for swine producers (5–7).

Four major PCV2 vaccines are licensed in Canada for pigs as young as 3 wk of age (3). This timing for vaccination can result in handling burdens for the producers and the piglets, particularly when the timing varies according to barn-specific protocols. Recent research showed that there was no significant difference in viral load or PCV2-associated lesions between PCV2-challenged piglets

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Table I. Experimental design

	Piglet age (wk)					
	0	1	2	3	4	5
		Early			Late	PCV2 challenge[b]
Treatment and group[a]		KLH[c] (n = 12)			KLH[c] (n = 10)	KLH (n = 12)
		VAC[d] (n = 11)			VAC[d] (n = 11)	VAC (n = 11)
						Never-VAC (n = 11)

[a] All piglets were monitored and sampled twice weekly. Quantitative PCV2-PCR, ELISA for anti-KLH antibodies, and virus neutralization assay (VNA) for PCV2-neutralizing antibodies were carried out on most serum and saliva samples. All statistical analysis were conducted using JMP 12 software (SAS Institute), applying a critical alpha.

[b] 10^5 $TCID_{50}$ PCV2 (intranasal).

[c] ~1.1 mg KLH in adjuvant per piglet (intramuscular).

[d] A single dose of Circumvent PCV was used.

that had been vaccinated as newborns (3 to 5 d of age) or piglets vaccinated at weaning (21 d of age) (7,8). Based on the demand from producers and these promising findings, a commercial vaccine was recently re-released with an additional label claim for use in piglets at 3 d of age (9). The efficacy of early vaccination is influenced by the maturity of the immune system. In swine, there is evidence of *in-utero* immune system maturation and class-switching from immunoglobulin M (IgM) to immunoglobulin G (IgG) without the influence of environmental antigens (10). Specifically, early developing piglets *in utero* (< 70 d) die upon amniotic exposure to porcine parvovirus, whereas later developing piglets (> 70 d) are able to mount a protective immune response and survive the parvovirus challenge (11). This transition at around 70 d of gestation coincides with maturation of the immune system (10) and suggests that piglets might be developmentally able to respond effectively to vaccination soon after birth.

This concept is supported by a recent study on humoral responses to early PCV2 vaccination using sows naive to PCV2 and PCV2 vaccination, thereby eliminating the chance of interference from PCV2-specific maternal antibodies (7,12). Piglets vaccinated early responded equally well to PCV2 vaccination and challenge as older vaccinees (7,12). Given that most commercial barns are currently PCV2-seropositive and the majority of gilts will have received at least 1, if not 2, PCV2 vaccines in their lifetime; however, it is important to understand maternal antibody effects, which are transferred from the sow to piglets in colostrum (12–15), in order for early vaccination to be translated into practice.

The current study was designed to determine the relationship between PCV2-antibody positive conditions, which are common in Canadian swine barns, and early vaccination with early PCV2 challenge. The hypotheses being tested are that piglets i) are equally able to produce antibodies to a novel antigen [keyhole limpet hemocyanin (KLH)] (16,17); and ii) respond equivalently to early and late PCV2 vaccination and challenge, resulting in similar serum viral load, salivary virus shedding, and PCV2-neutralizing antibody responses (nAb). If these hypotheses are supported by the results, the evidence base for translating early vaccination into industry practice will be strengthened.

Materials and methods

Animal source and housing

Five multiparous (3rd or 4th parity) sows (Genetiporc F25) and their 57 colostrum-fed piglets (aged 3 to 4 d; G-performer 4 boar as sire) were obtained from a western Canadian farm positive for PCV2. Sows were previously vaccinated against PCV2 with Ingelvac Circoflex at their weaning and again at their gilt selection. Throughout the study, animals were housed in a Biosafety Level-2 facility at the Veterinary Sciences Research Station of the University of Calgary, following modern pig production practices and biosafety and biosecurity protocols. Research was conducted with University of Calgary Animal Protocol Approval AC13-0150.

Five experimental groups

To ensure equal group size and to avoid bias due to different levels of maternal antibodies in the milk and genetic factors, groups were mixed from all 5 litters and each of the 5 groups was represented by at least 2 piglets from each litter. Two groups were immunized with a novel antigen, keyhole limpet hemocyanin (KLH) (16), that was mixed with the PCV2 vaccine, as detailed in this article. The Early-KLH group was immunized immediately after the first sampling in week 1 (6 to 7 d of age = days 0 to 2 of the study) and the Late-KLH group was immunized at the end of week 4 of sampling (27 to 28 d of age = days 21 to 23 of the study). Three additional groups were created to study the effects of early *versus* late PCV2 vaccination. The Early-VAC group was vaccinated against PCV2 after their first sampling in week 1 of the study, the Late-VAC group was vaccinated against PCV2 at the end of week 4 sampling, corresponding to industry-standard vaccination at > 3 wk of age, and the Never-VAC group received no treatment. At the end of week 5 (day 31), the Never-, Early-, and Late-VAC groups were exposed to PCV2 as described in the following sections and summarized in Table I.

Biosafety/security procedures

All piglets were handled and sampled weekly, from week 0 through week 10 of the study. Foot traffic among rooms of different

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treatment groups was minimized. Footbaths with Virkon S (DuPont, Wilmington, Delaware, USA) were placed outside each room. If a room was re-entered, new boots, coveralls, and barrier gloves were donned. Further cleaning and traffic protocols were put into place including using separate or completely cleaned equipment for each treatment group that could not be replaced, disinfecting the hallway after every sampling event, completely disinfecting and cleaning the surgery room where sampling took place, and consistent sampling of each treatment group by the same students.

Sampling procedures

Each piglet was physically examined immediately before every sampling procedure. Weight (± 0.1 kg), heart rate, respiration rate, and capillary refill time were determined and recorded for each piglet. Before each sample collection, piglets up to approximately 8 kg were anesthetized with isoflurane gas administered using a Bain non-rebreathing anesthetic system. When piglets were too large for the mask induction procedure, they were sedated with 3.3 mg/kg body weight (BW) of azaperone (Stresnil; Vétoquinol, Lavaltrie, Quebec) injected into the neck muscle (3 mL-syringe with a 22-gauge × 1-in needle) during sample collection.

For the first serum collection, 1 to 2 mL of blood was collected from the tail stub when tails were docked (by #10 scalpel) or from the auricular vein if the stub sample was insufficient. Subsequent samples were collected from the cephalic, median cubital, lateral saphenous, or auricular veins using 21- or 23-gauge butterfly catheters and a 3-mL syringe. When pigs were large enough to be sedated with azaperone, blood was collected from dorsally recumbent pigs from the cranial vena cava using 18 to 20 gauge × 1.5-inch needles with a Vacutainer system (BD Diagnostics, Mississauga, Ontario). Collected blood was allowed to clot at room temperature for about 1 h and then refrigerated at 4°C. After clot removal, serum was separated and stored in 1-mL aliquots at −80°C for subsequent analyses.

In addition to the blood samples, pooled piglet saliva was collected from the pens for Early-VAC, Late-VAC, and Never-VAC groups, 3 times a week (Tuesday, Wednesday, and Thursday) from weeks 6 through 10. Saliva was collected by placing a 30 cm, 3-strand, twisted cotton rope in each group's pen for 15 min (18). Piglets that explored the rope left saliva within the absorbent rope, which was extracted by wringing out (or twisting) the rope inside a clean ziplock bag, then decanting the liquid into a cryovial. Saliva samples were stored at −20°C for subsequent analyses.

Vaccination

Circumvent PCV vaccine (Intervet, Merck Animal Health, Summit, New Jersey, USA) was administered intramuscularly into the lateral aspect of the neck as a single 2-mL dose. The adjuvant of the product is Microsol Diluvac Forte (Merck Animal Health) in a base of mineral oil and dl-alpha-Tocopherol-acetate.

KLH antigen exposure

Keyhold limpet hemocyanin (KLH) was mixed with Circumvent PCV since the adjuvant of the vaccine could not be provided to us separately. Twenty milligrams of KLH (Sigma-Aldrich, St. Louis, Missouri, USA) were reconstituted in 5 mL of sterile water to a concentration of 4 mg/mL. Reconstituted KLH (3.75 mL, 15 mg) was added to 30 mL of Circumvent PCV vaccine in order to prepare 14 doses of 2.25 mL each. Treatments with KLH were administered intramuscularly into the left or right lateral aspect of the neck.

PCV2 challenge

A virus stock was derived from a molecular clone of PCV2 (Canadian strain 05-32650, GenBank accession no. EF394779), passaged several times through PK-15 cells, frozen and thawed 3 times, sonicated, and clarified at 400 × *g* for 5 min. The clarified stock was aliquoted and frozen at −80°C. Infectivity was 2.3×10^3 50% tissue culture infectious doses ($TCID_{50}$) per milliliter as determined by endpoint titration on PK-15 cells. The PCV2 challenge consisted of a 2-mL inoculum of PCV2 administered intranasally to a sedated pig restrained with the head elevated. The upright position was maintained for 5 s to ensure contact with the nasal mucosa. Excess liquid was then wiped from the snout and disposed of as biohazardous material.

KLH antibody ELISA

The KLH antibody enzyme-linked immunosorbent assay (ELISA) was developed in-house using a peroxidase labelled rabbit anti-pig IgG (whole molecule) (Sigma Aldrich) as the secondary antibody. Keyhole limpet hemocyanin (KLH) (Sigma Aldrich) was first reconstituted in sterile double-distilled water to a concentration of 4 mg/mL and then diluted in carbonate-bicarbonate buffer (1.5 mM, pH 9.6) to a final concentration of 4 ng/μL. ELISA plates were coated with 400 ng per well KLH, incubated overnight at 4°C, and washed 3 times with phosphate-buffered saline (PBS) with 0.1% (v/v) Tween (PBST). Wells were blocked with 2% (w/v) skim milk (from powder) in PBST at 100 μL per well for 1.5 h at room temperature and washed again 3 times. The positive control consisted of pooled serum samples from the Early-KLH group, week 5, at a 1:10 dilution in PBST. The negative control consisted of pooled serum samples from the Early-VAC group, which did not get KLH antigen, week 5, at a 1:10 dilution. The conjugate control was 2% skim milk in PBST.

The serum samples from the Early- and Late-KLH piglets were diluted in 4 steps from 1:20 to 1:160 in 2% skim milk and all dilutions were tested in duplicates by ELISA. Samples that still tested positive at 1:160 were titrated out to a maximum dilution of 1:800. A volume of 100 μL of diluted sample per well was incubated overnight at 4°C. Wells were then washed 3 times with PBST. The secondary antibody, peroxidase-labelled rabbit anti-pig IgG (whole molecule) (Sigma Aldrich), was diluted 1:10 000 2% milk in PBST. One hundred microliters of the dilute antibody were used per well and incubated for 1.5 h at 37°C in a cell culture incubator. Wells were then washed 3 times with PBST and developed with 2,2-azino-bis (ABTS) (100 μL per well, room temperature, 20 min in the dark). Absorbance (optical density, OD) was read using a Microplate Absorbance Reader ("iMark," Biorad, Mississauga, Ontario) with a 415 nm filter. Samples were run in duplicate, averaged, and reported as sample/positive ratios S/P = (sample OD — negative control OD)/(positive control OD — negative control OD). S/P values < 0.4 (mean + 2× SD of negative control samples) were scored as negative, while S/P values ≥ 0.4 were considered positive. The S/P values of positive samples were then translated to threshold titer values, i.e., the maximal dilution with a positive S/P value, and used in analyses.

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Serum and saliva viral load

All serum and saliva samples were tested for the presence of PCV2 genomic deoxyribonucleic acid (DNA) by real-time quantitative polymerase reaction (qPCR) using the PerfeCTa SYBR Green SuperMix with Low ROX (Quanta BioSciences, Gaithersburg, Maryland, USA) on a CFX96 detection system (Bio-Rad, Hercules, California, USA). Nucleic acid was extracted from 100 μL of serum or saliva with the Mag-Bind Viral DNA/RNA Kit (Omega Bio-Tek, Norcross, Georgia, USA) on a MagMAX Express-96 magnetic particle processor (Life Technologies, Burlington, Ontario) (4). All tests were conducted in duplicate and only samples that were positive in both replicates were considered positive.

Serum PCV2-neutralizing antibodies (PCV2-nAb)

After 1 h of heat inactivation at 56°C, serum samples were serially diluted 3-fold in PBS, mixed with PCV2 (2.3×10^3 $TCID_{50}$/well), incubated at 37°C for 1 h, and added to 96-well plates containing PK-15 cell monolayers seeded the previous day. After incubation on the cells for 90 min at 37°C, the supernatant was removed, cells were washed with sterile PBS, and 100 μL of fresh media were added. Plates were incubated at 37°C for 48 h. For indirect immunofluorescence staining, the supernatant was discarded, cells were washed with PBS and fixed with 4% (w/v) formaldehyde (from paraformaldehyde powder) in PBS at room temperature for 30 min and subsequently washed again with PBS. Fixed cells were treated with permeabilization buffer [PBS with 0.1% bovine serum albumin (BSA) and 0.1% (v/v) saponin] and then incubated with primary antibody (rabbit anti-PCV2-Cap) diluted in PBS followed by secondary antibody (goat anti-rabbit Alexa Fluor 568) and 4′6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature. Plates were then analyzed using an automated fluorescence microscope (IN Cell Analyzer 2000; GE Healthcare, Mississauga, Ontario). Titers were defined as the highest serum dilution that reduced viral infectivity by 50% (4,19).

Statistical analyses

All statistical analyses were conducted using JMP 12 software (SAS Institute, Cary, North Carolina, USA), applying a critical alpha of 0.05. As detailed in the text for each test, non-parametric Wilcoxon and Kruskal-Wallis tests were applied to data sets with non-normal distributions, followed by *post-hoc* Chi-squared approximation or comparison against control (Dunn all pairs for joint ranks) as appropriate. Parameters with a normal distribution were analyzed using *t*-test or by analysis of variance (ANOVA) with *post-hoc* Tukey-Kramer as appropriate. Measures of titer were converted to log [titer + 1] so that titer = 0 was retained as data = 0, rather than being excluded from analyses as missing data.

Results

Piglet exclusion and inclusion

The 5 litters consisted of a total of 57 piglets (2 × 12, 3 × 11 piglets). One piglet in the Never-VAC group showed signs of PCVAD and was euthanized in week 9. Necropsy findings of this piglet included generalized lymphoid depletion and lesions consistent with acute streptococcal septicemia. All data for this piglet were retained in analyses. A second piglet assigned to the Early-VAC group was lost to sow rollover in week 1 and was excluded from all analyses. A third piglet in the Late-KLH group with acute lameness, fever, and neurological signs was euthanized in week 7, after failure to respond to treatment for *Streptococcus suis*. Postmortem diagnosis was polyserositis. This piglet was excluded from all analyses as the disease might have affected the immune response to KLH. In total, results for 55 piglets were included in the analyses.

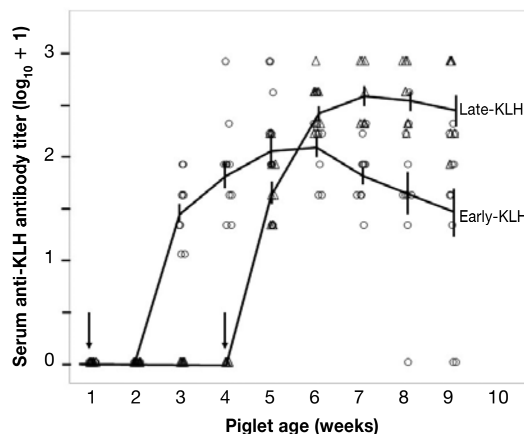

Figure 1. Antibody response to immunization with keyhole limpet hemocyanin (KLH). Piglets were immunized with KLH at 6 to 7 (○) and 27 to 28 (△) d of age, as indicated by vertical arrows. KLH-specific IgG titers are shown as the base-2 logarithm after adding 1 to the original values to demonstrate a base line at 0. Vertical bars represent the standard error of the mean (SEM) of a given group on a specific sampling day. Means of each group are connected by a line.

Health status

There were no significant differences in initial piglet weight across groups in week 1 ($F4, 55 = 1.86$, $P = 0.13$). There were also no significant differences between control piglets (Never-VAC) and any other group in week 1 or week 9 ($P = 0.23$; $P = 0.06$). Weight gain from weeks 3 to 9 (0.43 ± 0.08 kg/d) was consistent with expectations for nursery pigs in the US from 2005 to 2010 (0.38 ± 0.05 kg/d from day 19 to day 65). With a mean piglet temperature of 38.9°C ± 0.1°C and a range from 37.4°C to 39.9°C, there was no evidence of hyperthermia (> 40°C) at the onset of the study and no significant difference among treatment groups ($F4, 53 = 0.96$, $P = 0.50$).

Antibody response to KLH

No antibodies to KLH were detected in animals in either the Early-KLH or the Late-KLH group before exposure to KLH antigen. All piglets immunized with KLH eventually developed specific antibodies (Figure 1). Specifically, at 1 wk after KLH antigen exposure, piglets in the Early-KLH group showed no KLH antibodies, whereas all piglets in the Late-KLH group had a positive KLH antibody titer, resulting in a significant difference between early and late exposure (Wilcoxon Chi-square approximation = 15.84, df = 1, $P < 0.0001$). By the second week post-immunization, all piglets were positive for KLH antibodies. In weeks 2 and 3 after exposure, the Late-KLH group

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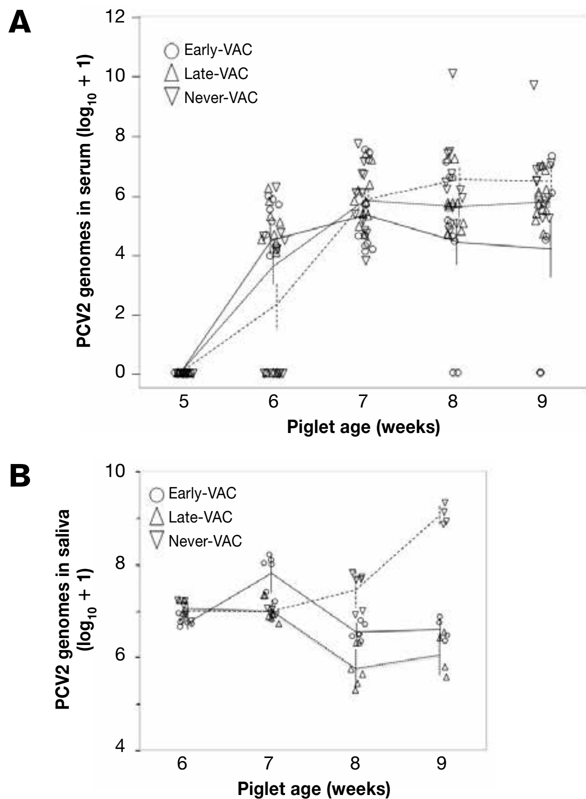

Figure 2. PCV2 genome load in serum (A) and saliva (B). Viral load is shown for piglets from Early-VAC (○), Late-VAC (△), and Never-VAC (▽) groups. PCV2 genome copy numbers from qPCR are shown as the base-10 logarithm after adding 1 to the original values to demonstrate a base line at 0. Pooled saliva was collected from each group 3 times a week and quantified in duplicate, with the mean qPCR copy number presented for each sample. Vertical bars represent the standard error of the mean (SEM) on a given group at a specific sampling day. Means of each group are connected by a line.

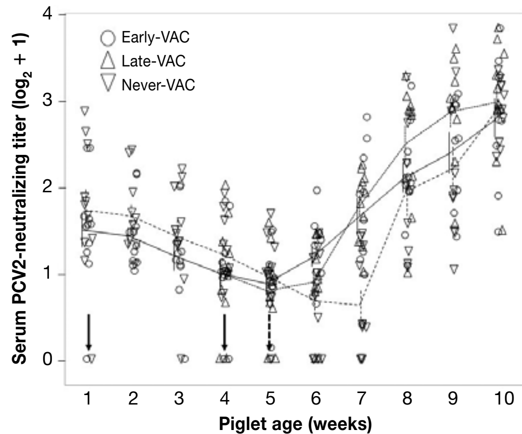

Figure 3. Development of serum PCV2-neutralizing antibodies. Titers of PCV2-neutralizing IgG (nAb) are shown for piglets from Early-VAC (○), Late-VAC (△), and Never-VAC (▽) groups. Vertical arrows indicate day of immunization (solid) and of PCV2 challenge (dashed). Titers of nAb are shown as the base-2 logarithmic after adding 1 to the original values to demonstrate a base line at 0. Vertical bars represent the standard error of the mean (SEM) on a given group at a specific sampling day. Means of each group are connected by a line (Early-VAC — solid line; Late-VAC — narrow dashes; Never-VAC — wider dashes).

(older at immunization) continued to demonstrate a stronger antibody response than the Early-KLH group (younger) (both $P < 0.005$). In week 4 after exposure, a significant but smaller difference remained ($P < 0.025$). However, the last comparable time-point (5 wk after the KLH exposure) did not show a significant difference between the 2 groups ($P = 0.09$). Thus, both groups achieved equivalent antibody titers against the KLH antigen within 5 wk.

PCV2 genome load in serum

Sera from both the Early- and Late-KLH groups were negative in the PCV2 qPCR over the entire course of the study. All 3 PCV2 groups remained negative up to and including the day before they were exposed to PCV2 (after the week 5 sample). This confirmed the effectiveness of our biosafety procedures. One week (5 or 6 d) after PCV2 challenge, 24/34 piglets were positive for PCV2 in the serum by qPCR (Figure 2A), with 1 Early-VAC (sampled 6 d post-challenge), 3 Late-VAC (5 d post-challenge), and 6 Never-VAC (6 d post-challenge) piglets negative. By week 2 after challenge (age 6 wk), sera from all (34/34) piglets were positive in the PCV2 qPCR. There was no difference between the PCV2 genome copy numbers in sera from Never-VAC, Early-VAC, and Late-VAC piglets (Kruskal-Wallis, Chi-square approximation, df = 2, $P > 0.4$) in weeks 2 to 4 post challenge. Over weeks 8 and 9, 3 piglets (5 samples) had no detectable virus genome in serum, although the same piglets had been positive in week 7. All samples with no detectable virus genome came from the Early-VAC group. The 1 piglet with the highest viral load in weeks 8 and 9 of the Never-VAC group was later euthanized, but retained in analyses. Thus, all challenged piglets became positive for PCV2 genome in serum, 1 progressed to clinical disease and death, and 3 reverted to qPCR-negative status before the end of the study.

Virus shedding in saliva

The PCV2 genome load in pooled saliva was determined weekly, from weeks 6 through 9, based on 3 samples per week from the Early-, Late-, and Never-VAC pens. Across treatment groups, piglets from the Never-VAC group shed significantly more PCV2 than those from the Late-VAC group (ANOVA with *post-hoc* Tukey-Kramer testing $P < 0.005$). No overall effect of week was seen ($P = 0.39$). Within week 7, shedding of PCV2 by Early-VAC piglets exceeded that of Late-VAC and Never-VAC piglets ($P < 0.05$). Within weeks 8 and 9, Never-VAC piglets had higher PCV2 genome loads in saliva than those from both Early- and Late-VAC groups (both $P < 0.005$) (Figure 2B). Thus, vaccination reduced virus shedding in saliva compared to the Never-VAC group, although the Early-VAC group did have a phase of higher shedding.

PCV2-neutralizing antibodies in serum

All piglets had detectable levels of PCV2-neutralizing antibodies in the first week of sampling and before PCV2 vaccination. These antibodies were regarded as transferred from mothers through colostrum and their titers declined until exposure to PCV2 at day 32 of the study (Figure 3). Vaccination, whether at an early or older age, did not induce substantial amounts of PCV2-neutralizing antibodies before PCV2 challenge.

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All animals challenged with PCV2 showed increasing neutralizing antibody levels across kinetics of PCV2-neutralizing antibodies after PCV2 challenge ($P < 0.0001$), indicating that neither age nor presence of maternal antibodies interfered with development of neutralizing antibody in vaccinated piglets. In week 7, piglets in the Never-VAC group had a significantly lower titer than both the Early- and Late-VAC piglets (both $P < 0.001$). Thus, the Never-VAC group was delayed by at least 1 wk in mounting a neutralizing antibody response compared to both groups that were vaccinated. Once the Never-VAC piglets began to respond with the production of PCV2-nAB, they rapidly caught up to the Early-VAC piglets.

Discussion

This experimental study was conducted to determine whether piglets could be successfully vaccinated against PCV2 at a very young age and whether maternal antibodies would interfere with vaccination efficacy. Our results indicate that PCV2 vaccination of piglets less than 6 days old produced results similar to those obtained from older vaccinees. As reported in a previous study, however, PCV2-neutralizing antibodies were not induced by vaccination *per se* but only upon infection with PCV2 (4). Further study is required to determine whether this lack of induction of PCV2-neutralizing antibody resulted from a weakness of the PCV2 vaccine formulation and/or from circulating maternal antibodies against PCV2 that inhibited the vaccination. Certainly, the lack of suitable levels of neutralizing antibodies before PCV2 exposure is a major reason for the high prevalence of PCV2 on many pig farms today (4).

Maturity of immune system in newborn piglets

It has been demonstrated in a previous study that PCV2 vaccination of less than 6-day-old piglets is as efficacious as vaccination of older pigs (7). It was thus not surprising to find antibody responses of KLH vaccinees immunized early in life to be very similar to those of older vaccinees. Since our enzyme-linked immunosorbent assay (ELISA) detected exclusively IgG, we can only assume that a class switch from IgM to IgG occurred very soon after KLH immunization. The kinetics of the humoral immune response and the inferred immunoglobulin class switch indicate that pigs already possess a mature immune system at birth. Our data extend previous findings (7), but further studies are needed to determine whether the immune system of younger pigs requires similar or different amounts of immunogen and/or adjuvant than older pigs to be equally efficacious.

Effects of maternal antibodies

We found substantial levels of PCV2-neutralizing antibody in almost all newborn piglets after ingestion of colostrum. These antibodies waned over a period of approximately 5 wk (Figure 3). As all sows of our experimental piglets had been vaccinated against PCV2 at least twice, these perinatal antibodies most likely represent maternal antibodies. It has been suggested that maternal antibodies protect piglets from PCVAD but not entirely from PCV2 infection (20,21). On the other hand, it is not clear whether maternal anti-PCV2 antibodies interfere with PCV2 vaccination as occurs with various other vaccinations, both in humans and animals [for review, see (22)]. Opriessnig et al (8) found no evidence that maternal antibodies inhibited PCV2 vaccination. Only 1 commercial vaccine was tested in that study, however, and outcomes could vary depending on the type of vaccine used.

Our results indicate that generation of PCV2-neutralizing antibodies was very similar in young and old vaccinees. Notably, most of the young vaccinees had maternal PCV2-specific antibodies in their sera at the time of vaccination and were thus immunized in the presence of anti-PCV2 antibodies. Our data indicate that maternal antibodies did not interfere with PCV2 vaccination under the given circumstances. While these results are concordant with published data (8), they would require higher statistical power for a conclusive statement about whether maternal antibodies do or do not inhibit PCV2 vaccination.

Efficacy of vaccination

The ultimate goal of prophylactic vaccination is prevention of infection. Since all pigs became infected after exposure to PCV2, this goal was not met. Our data do not differ from those of numerous other experimental and clinical studies and highlight a profound weakness of current PCV2 vaccines. However, all currently commercially available PCV2 vaccines do reliably prevent PCVAD (23). We assessed the efficacy of PCV2 vaccination by determining the levels of PCV2 genomes in sera and saliva and by the *de novo* generation of PCV2-neutralizing antibody. We observed no difference between the PCV2 genome copy numbers in sera from Never-VAC, Early-VAC, and Late-VAC piglets in weeks 2 to 4 post challenge. Only piglets in the Early-VAC treatment group had undetectable PCV2 viremia by the end of the study and those piglets also fell at the low end of copy numbers relative to other piglets in the previous week. It is therefore possible that the longer interval between vaccination and challenge (4 wk *versus* 1 wk) enhanced the booster effect of the PCV2 challenge. Our study allowed only 1 wk for the Late-VAC piglets to respond to vaccination before challenge. As a result, our study simulated the risk of PCV2 exposure in early life and was able to identify benefits of early vaccination.

Porcine circovirus type-2 (PCV2) is shed and transmitted *via* oronasal secretions. During the final 2 wk of our experiment, virus shedding in saliva was significantly lower in the 2 vaccinated groups than in the Never-VAC group, in which shedding continued to increase over time. Again, this result demonstrated that vaccination of both young and older piglets was equally efficacious. This is further supported by the kinetics of PCV2-neutralizing antibody production. After viral challenge, all groups and piglets developed higher PCV2-neutralizing titers. The groups were similar in week 6, 1 wk after challenge, and were also similar in weeks 8 through 10, which indicates that the cumulative immune response to PCV2 challenge was the same in the presence or absence of vaccination and confirms the findings of O'Neill et al (7) that early vaccination does not alter the neutralizing antibody response relative to later vaccination. The Never-VAC piglets did not increase their neutralizing capacity in serum between weeks 6 and 7, however, which resulted in a 1-week delay in the response of the Never-VAC piglets. Thus, vaccination did accelerate the development of the neutralizing antibody response (24), but was not necessary for a strong response over time.

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In conclusion, the findings of this study were complementary in many ways to those of O'Neil et al (7). Both studies found strong evidence of a humoral response to PCV2 vaccination in the first week of life and confirmed that the responses of early- and late-vaccinated piglets tend to converge over time. The current study adds evidence that exposure to KLH, a novel antigen, follows a similar time course. With earlier experimental infection in the current study, these data therefore suggest that early vaccination reduces virus shedding in saliva and is preferable to traditional vaccination if the infectious challenge is likely to be experienced quite early in life.

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Article

Evaluation of the new commercial recombinant chimeric subunit vaccine PRRSFREE in challenge with heterologous types 1 and 2 porcine reproductive and respiratory syndrome virus

Jiwoon Jeong, Changhoon Park, Kyuhyung Choi, Chanhee Chae

Abstract

The objective of this study was to evaluate a new recombinant chimeric vaccine against porcine reproductive and respiratory syndrome virus (PRRSV). The subunit vaccine, PRRSFREE, from Reber Genetics, Taiwan, Republic of China, is based on a plasmid containing a detoxified *Pseudomonas* exotoxin carrying open reading frame (ORF) 7, 1b, and 5 and 6 chimeric subunits of types 1 and 2 PRRSV. Pigs were injected intramuscularly with 2.0 mL of the vaccine at 21 and 42 d of age, according to the manufacturer's recommendation. At the age of 63 d the pigs were inoculated intranasally with either type 1 or type 2 PRRSV. Regardless of the genotype of the challenging PRRSV, the vaccinated challenged pigs had significantly lower ($P < 0.05$) mean rectal temperature, respiratory score, lung lesion score, and amount of PRRSV antigen within areas of interstitial pneumonia, along with overall lower levels of viremia due to type 1 or type 2 PRRSV compared with the unvaccinated challenged pigs. The vaccinated challenged pigs also had significantly higher ($P < 0.05$) numbers of interferon-γ secreting cells compared with the unvaccinated challenged pigs. This study demonstrated that the new vaccine provides protection against respiratory disease from heterologous types 1 and 2 PRRSV challenge in growing pigs.

Résumé

L'objectif de la présente étude était d'évaluer un nouveau vaccin recombinant chimérique contre le virus du syndrome reproducteur et respiratoire porcin (VSRRP). Le vaccin sous-unitaire, PRRSFREE, de Reber Genetics, Taïwan, République de Chine, est basé sur un plasmide contenant une exotoxine de Pseudomonas *détoxifiée portant les cadres de lecture ouverts (ORF) 7, 1b, et 5 et 6 unités chimériques des types 1 et 2 du VSRRP. Les porcs ont été injectés par voie intramusculaire avec 2,0 mL du vaccin à 21 et 42 j d'âge, selon les recommandations du manufacturier. À l'âge de 63 j les porcs ont été inoculés par voie intra-nasale avec le type 1 ou le type 2 du VSRRP. Indépendamment du génotype du VSRRP utilisé pour l'infection défi, des données significativement moindres* (P < 0,05) *étaient notées chez les porcs vaccinés et infectés comparativement aux animaux non-vaccinés et infectés quant à la température rectale moyenne, le pointage respiratoire, le pointage de lésions pulmonaires, et la quantité d'antigènes du VSRRP dans les sites de pneumonie interstitielle, de même que pour le degré de virémie dû au type 1 ou au type 2 du VSRRP. Les porcs vaccinés et challengés avaient également une quantité significativement plus grande* (P < 0,05) *de cellules secrétant de l'interféron-γ comparativement aux porcs non-vaccinés et infectés. Cette étude démontre chez des porcs en croissance que le nouveau vaccin fourni une protection contre les problèmes respiratoires causés par une infection défi hétérologue avec les types 1 et 2 du VSRRP.*

(Traduit par Docteur Serge Messier)

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first reported in 1987 in the United States and had become widespread throughout Europe by 1991 (1). During the mid-1990s PRRS was also reported in Asian countries (2–4). The most economically important disease facing the global swine industry, PRRS occurs in 2 forms: reproductive failure in pregnant sows and respiratory disease in pigs of all ages (5). The causative agent is the PRRS virus (PRRSV), an enveloped positive-strand RNA virus classified in the order *Nidovirales*, family *Arteriviridae*, and genus *Arterivirus* (6). The PRRSV genome contains at least 9 open reading frames (ORFs), which encode the viral replicases (ORF1a and 1b) and 7 structural proteins (ORF2a, 2b, 3, 4, 5, 6, and 7) (6).The virus falls into 2 distinct genotypes, referred to as type 1 (European) and type 2 (North American), which are genetically and antigenically distinct (7–9).

Asian PRRSV isolates were of type 2 until 2000 (10). Early that year, type 1 PRRSV emerged in Asian countries (11–13). Currently both genotypes are circulating in Asian pig farms. Therefore, protection by vaccine against both genotypes is an important clinical issue in many Asian countries because of a lack of cross-protection by current vaccines against heterotypic PRRSV (14–16). A new

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Table I. Experimental design for evaluation of a new recombinant chimeric vaccine against porcine reproductive and respiratory syndrome virus (PRRSV) in pigs vaccinated (Vac) or not vaccinated (UnVac) at 21 and 42 d of age and then challenged at 63 d of age with type 1 (Ch1) or type 2 (Ch2) PRRSV or not challenged (UnCh)

Group	Vaccinated	Challenged with PRRSV type 1 or 2
Vac/Ch1	Yes	Type 1
UnVac/Ch1	No	Type 1
Vac/Ch2	Yes	Type 2
UnVac/Ch2	No	Type 2
Vac/UnCh	Yes	No
UnVac/UnCh	No	No

recombinant chimeric PRRS vaccine (PRRSFREE PRRS subunit vaccine) was introduced into the Taiwanese market in 2012 by Reber Genetics (Taiwan, Republic of China), which claimed that it protected against both genotypes. Since the current needs in a PRRS vaccine are better safety and better efficacy against both genotypes (17), this new vaccine fit perfectly with the demands of swine producers. Theoretically, the vaccine has the potential to be clinically useful in controlling coinfection by both genotypes. However, there had been no reports on scientific studies in peer-reviewed publications that demonstrated protection of this vaccine against either PRRSV genotype. The objective of this study, therefore, was to evaluate the efficacy of the new vaccine in controlling respiratory disease in growing pigs challenged with heterologous types 1 and 2 PRRSV on the basis of clinical, immunologic, virologic, and pathological evaluations under experimental conditions.

Materials and methods

Vaccine

The recombinant chimeric PRRS vaccine used in this study is based on a plasmid containing a detoxified *Pseudomonas aeruginosa* exotoxin and ORF7, ORF1b, ORF5, and ORF6 chimeric subunits of type 1 and type 2 PRRSV (18). The epitopes of viral ORF7 and ORF1b are made from the conserved regions of PRRSV and other arteriviruses [GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) no. X53459 for *Equine arteritis virus*, M96262 for PRRSV, U15146 for *Lactate dehydrogenase-elevating virus*, and U63121 for *Simian hemorrhagic fever virus;* US patent no. 7,595,054 B2]. Viral ORF5 and ORF6 are made from type 1 PRRSV (GenBank no. CAA63493.1) and type 2 PRRSV (GenBank ACG52416.1), respectively. The ORF1b gene encodes the key enzymes for PRRSV RNA synthesis, which is essential for genome replication and synthesis (19). The PRRSV subunit antigens encoded by ORF5 through ORF7 have been reported to confer immunogenicity (20,21). The GP5 protein encoded by ORF5 and the M protein encoded by ORF6 can induce neutralizing antibodies (22,23). The efficacy of PRRSV DNA vaccination is significantly enhanced by coexpression of the GP5 and M proteins as heterodimers (24). Therefore, a new recombinant chimeric vaccine containing ORF1b and ORFs 5 to 7 is a good choice to control PRRSV infection.

Virus inocula

The type 1 PRRSV SNUVR090485, a pan-European subtype 1 (GenBank no. JN315686), and the type 2 PRRSV SNUVR090851, lineage 1 (GenBank no. JN315685), were used as inocula. The type 1 virus was isolated from lung samples from an aborted fetus from southwestern Gyeonggi Province in 2009 (25). The type 2 virus was isolated from lung samples from newly weaned pigs in Chungcheung Province in 2010 (26). The 2 viruses have 59% ORF5 nucleotide identity.

Experimental design

A total of 108 colostrum-fed, cross-bred, conventional piglets were purchased at 14 d of age from a commercial PRRSV-free farm. All the piglets were negative for PRRSV, porcine circovirus 2 (PCV2), and swine influenza virus according to routine serologic testing, and all the piglets were negative for types 1 and 2 PRRSV viremia according to real-time polymerase chain reaction (PCR) (27).

The pigs were divided into 6 groups (18 pigs per group) by means of random number generation with Excel (Microsoft Corporation, Redmond, Washington, USA) (Table I). Those in groups Vac/Ch1, Vac/Ch2, and Vac/UnCh were injected intramuscularly in the right side of the neck with 2.0 mL of PRRSFREE (lot F4001) at 21 and 42 d of age, according to the manufacturer's recommendation. The pigs in groups UnVac/Ch1, UnVac/Ch2, and UnVac/UnCh were injected intramuscularly in the same anatomic location with 2.0 mL of phosphate-buffered saline (0.01 M, pH 7.4) at the same age. At the age of 63 d the pigs in groups Vac/Ch1 and UnVac/Ch1 were inoculated intranasally with 3 mL of tissue culture fluid containing 10^5 50% tissue culture infective doses ($TCID_{50}$)/mL of type 1 PRRSV (SNUVR090485, 2nd passage in alveolar macrophages). The pigs in groups Vac/Ch2 and UnVac/Ch2 were inoculated intranasally with 3 mL of tissue culture fluid containing 10^5 $TCID_{50}$/mL of type 2 PRRSV (SNUVR090851 strain, 2nd passage in MARC-145 cells). The pigs in the UnVac/UnCh group remained unvaccinated and unchallenged and served as negative controls. The pigs in each group were housed separately within the facility. Blood samples and nasal swabs were collected at −35, −28, −21, −14, −7, 0, 3, 7, 10, 14, and 21 d after challenge. Subsets of pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 7, 14, and 21 d after challenge as previously described (28). Tissues were collected from each pig at necropsy. All of the methods had been approved by the Seoul National University Institutional Animal Care and Use and Ethics Committee.

Clinical observation

The pigs were monitored weekly for physical condition and scored daily for clinical severity of respiratory disease from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (29). Rectal temperature was recorded daily at the same time by the same personnel.

Quantification of PRRSV RNA

RNA was extracted from the blood samples and nasal swabs to determine the numbers of genomic RNA copies for types 1 and 2

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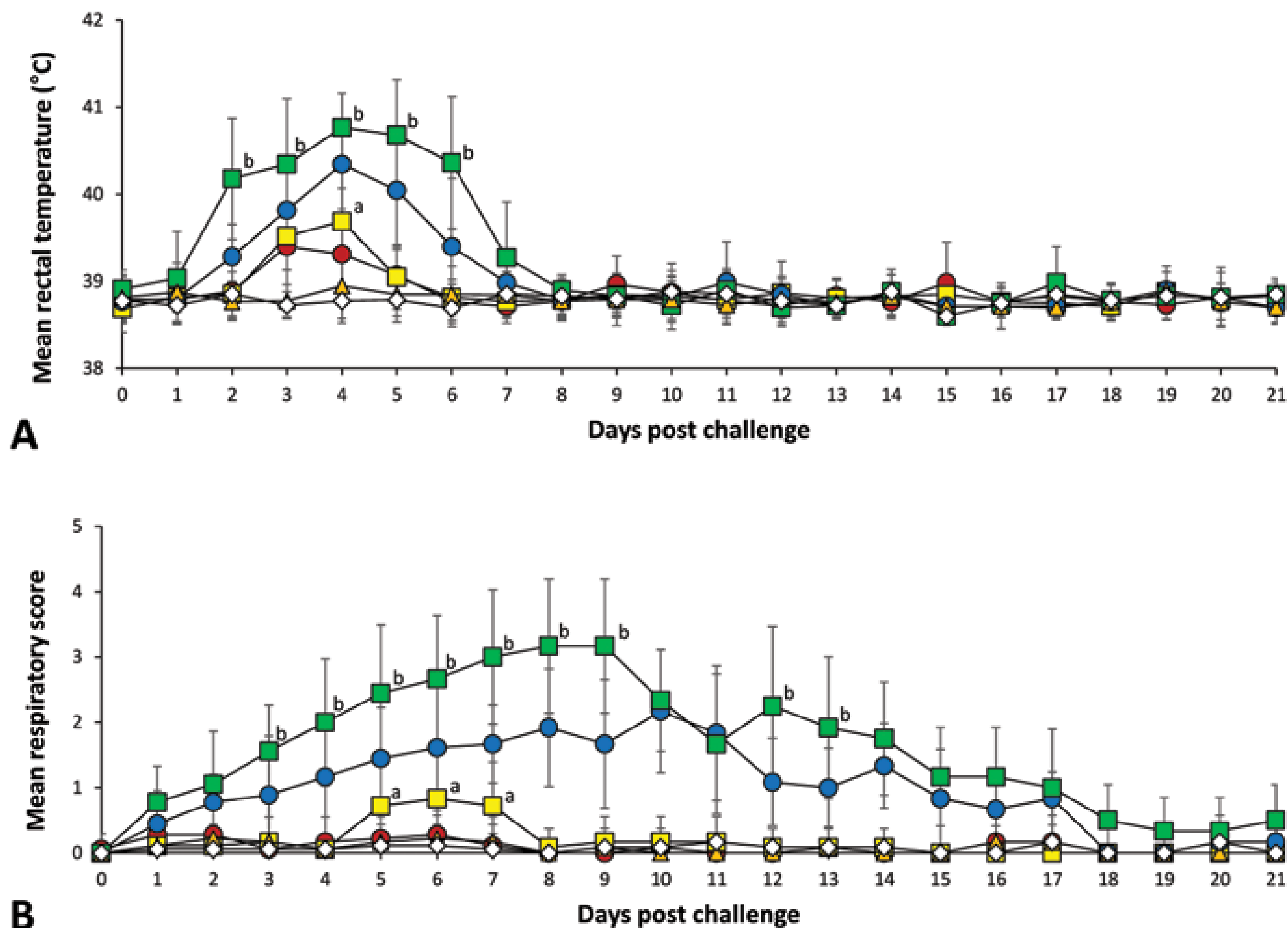

Figure 1. Mean rectal temperature (A) and mean respiratory score (B) of pigs vaccinated (Vac) or not vaccinated (UnVac) with a new recombinant chimeric vaccine against porcine reproductive and respiratory syndrome virus (PRRSV) and then challenged with type 1 (Ch1) or type 2 (Ch2) PRRSV or not challenged (UnCh). Symbols as follows: Vac/Ch1 (●), UnVac/Ch1 (■), Vac/Ch2 (●), UnVac/Ch2 (■), Vac/UnCh (▲), and UnVac/UnCh (◇). Variation is expressed as the standard deviation. [a,b] Denote significant differences ($P < 0.05$) between Vac/Ch1 and UnVac/Ch1[a] and between Vac/Ch2 and UnVac/Ch2[b] on the same day after challenge.

PRRSV by real-time PCR as previously described (27,30). Sterile polyester swabs (Fisher Scientific, Pittsburgh, Pennsylvania, USA) had been used to reach deeply into the turbinates to swab the nasal mucosa of both nostrils. The swabs had been stored in 5-mL plastic tubes (Fisher Scientific) containing 1 mL of sterile saline solution.

Antibody studies

Serum from the blood samples was tested with the commercial PRRSV enzyme-linked immunosorbent assay (ELISA) HerdChek PRRS X3 Ab test (IDEXX Laboratories, Westbrook, Massachusetts, USA). Samples were considered positive for PRRSV antibody if the sample/positive (S/P) ratio was greater than or equal to 0.4, according to the manufacturer's instructions.

Tests were also done to determine the titers of serum virus-neutralizing (VN) antibody against the challenge strains of PRRSV as previously described (31). The presence or absence of virus-specific cytopathic effect in each well was recorded after incubation for 7 d. Serum samples were considered to be positive for VN antibody if the titer was greater than 2.0 ($\log_2$) (32).

Interferon gamma studies

The numbers of PRRSV-specific interferon gamma (IFN-γ)-secreting cells (IFN-γ-SCs) were determined in peripheral blood mononuclear cells (PBMCs) by enzyme-linked immunospot assay (ELISPOT) as previously described (30,33,34).

The phenotypes of the $CD4^+CD8^+IFN\text{-}\gamma^+$ cells in PBMCs were analyzed as described elsewhere (31,35) by means of flow cytometry with the use of 3 monoclonal antibodies: CD4a against swine antigen conjugated with R-phycoerythrin (SouthernBiotech, Birmingham, Alabama, USA), CD8a conjugated with fluorescein isothiocyanate (SouthernBiotech), and IFN-γ conjugated with Alexa Fluor 647 (BD Biosciences, San Diego, California, USA).

Lung-tissue studies

The scores for macroscopic and microscopic lung lesions were analyzed morphometrically as previously described (29). *In-situ* hybridization for the detection and differentiation of types 1 and 2 PRRSV nucleic acids in the lung tissue was done, and the results were analyzed morphometrically as previously described (26).

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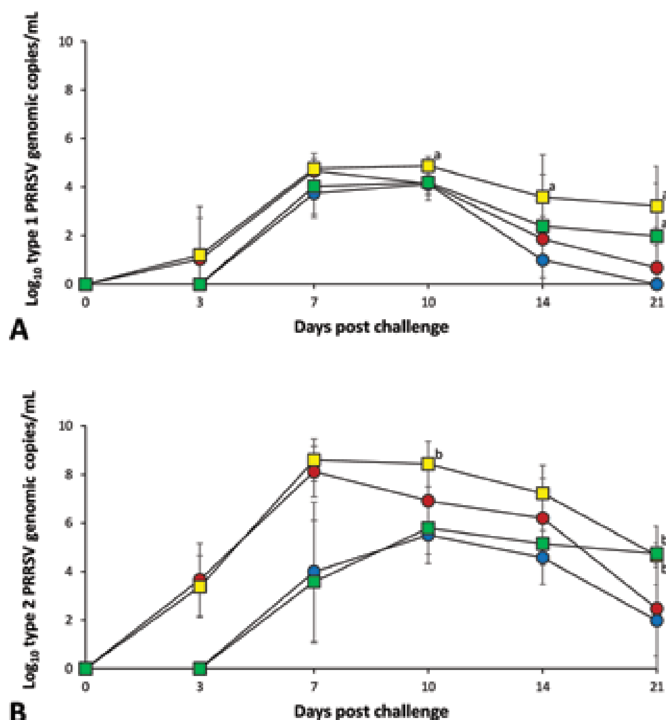

Figure 2. Mean numbers of genomic copies of type 1 PRRSV (A) and type 2 PRRSV (B) in the serum (● and ■) and nasal samples (● and ■) from pigs in the Vac/Ch1 (● and ●), UnVac/Ch1 (■ and ■), Vac/Ch2 (● and ●), and UnVac/Ch2 (■ and ■) groups. Variation and superscript letters (a,b) as for Figure 1.

The PRRSV was isolated from lung tissues with the use of alveolar macrophages for type 1 PRRSV and MARC-145 cells for type 2 PRRSV as previously described (29).

Statistical analysis

All real-time PCR and VN antibody data were transformed to $\log_{10}$ and $\log_2$ values, respectively. Summary statistics were then calculated for all of the groups to assess the overall quality of the data, including normality. The continuous data (for rectal temperature, PRRSV RNA, and serologic and PBMC findings) were analyzed by repeated-measures analysis of variance (ANOVA) for each time point; when significance was revealed, a 1-way ANOVA was done to determine the significance of individual between-group differences. Discrete data (scores for clinical respiratory disease, macroscopic and microscopic lung lesions, and PRRSV antigen) were analyzed by Mann–Whitney tests. Chi-square and Fisher's exact test were applied to evaluate the proportions of viremic pigs. A value of $P < 0.05$ was considered significant.

Results

The pigs in the UnVac/Ch1 group had significantly higher ($P < 0.05$) mean rectal temperatures (38.7°C to 39.7°C) than the pigs in the Vac/Ch1, Vac/UnCh, and UnVac/UnCh groups 4 d after challenge (Figure 1A). The pigs in the UnVac/Ch2 group had significantly higher ($P < 0.05$) mean rectal temperatures (39.5°C to 40.4°C) than the pigs in the Vac/Ch2, Vac/UnCh, and UnVac/UnCh groups on days 2 to 6 d after challenge (Figure 1A). The mean respiratory scores were significantly higher ($P < 0.05$) in the pigs in the UnVac/Ch1 group than in the pigs in the Vac/Ch1 group on days 5, 6, and 7 after challenge (Figure 1B). The mean respiratory scores were significantly higher ($P < 0.05$) in the pigs in the UnVac/Ch2 group than in the pigs in the Vac/Ch2 group on days 3, 4, 5, 6, 7, 8, 9, 12, and 13 after challenge (Figure 1B). The negative-control pigs (UnVac/UnCh) maintained normal temperatures without respiratory signs throughout the experiment.

Genomic copies of the type 1 PRRSV were detected in the serum of the pigs in the Vac/Ch1 and UnVac/Ch1 groups after inoculation with the virus, the number of copies being significantly lower ($P < 0.05$) in the former compared with the latter on days 10, 14, and 21 after challenge. The pigs in the Vac/Ch1 group also had a significantly lower ($P < 0.05$) number of genomic copies of type 1 PRRSV in the nasal swabs taken on day 21 after challenge compared with the pigs in the UnVac/Ch1 group (Figure 2A). Genomic copies of the type 2 PRRSV were detected in the serum of the pigs in the Vac/Ch2 and UnVac/Ch2 groups after inoculation with the virus, the number of copies being significantly lower ($P < 0.05$) in the former compared with the latter on days 10 and 21 after challenge. The pigs in the Vac/Ch2 group also had a significantly lower ($P < 0.05$) number of genomic copies of type 2 PRRSV in the nasal swabs taken on day 21 after challenge compared with the pigs in the UnVac/Ch2 group (Figure 2B).

The prevalence of viremia among the pigs is summarized in Table II. No type 1 PRRSV was detected in any serum or nasal sample from the type 2 PRRSV-challenged pigs and vice versa. No type 1 or type 2 PRRSV was detected in the serum and nasal samples from the negative-control pigs throughout the experiment.

The pigs in all 6 groups were seronegative for antibodies against PRRSV at the time of the 1st vaccination against PRRS, at 3 wk of age (−35 d after challenge). Antibodies specific for PRRSV were detected by ELISA in the pigs in the 3 vaccinated groups from day 3 after challenge onward. The titers were significantly higher ($P < 0.05$) in the Vac/Ch1 and Vac/Ch2 groups than in the UnVac/Ch1 and UnVac/Ch2 groups from days 3 to 21 after challenge (Figure 3). Anti-PRRSV antibody was not detected in the negative-control (UnVac/UnCh) pigs at any time in the experiment.

The titers of specific VN antibody against type 1 PRRSV were significantly higher ($P < 0.05$) in the Vac/Ch1 group than in the UnVac/Ch1 and Vac/UnCh groups at 14 and 21 d after challenge (Figure 4A). Similarly, the titers of specific VN antibody against type 2 PRRSV were significantly higher ($P < 0.05$) in the Vac/Ch2 group than in the UnVac/Ch2 and Vac/UnCh groups at 14 and 21 d after challenge (Figure 4B). Among the negative-control pigs VN antibody was not detected (titer $< 2 \log_2$) at any time in the experiment.

After stimulation by vaccination the number of PRRSV-specific IFN-γ-SCs per 10^6 PBMCs reached an average of 22.2 ± 18.9 in the pigs in the Vac/Ch1 group and 29.6 ± 8.6 in the pigs in the Vac/Ch2 group at −7 d after challenge. Upon challenge with PRRSV the numbers of PRRSV-specific IFN-γ-SCs per 10^6 PBMCs increased gradually and reached an average of 61 ± 20.2 in the pigs in the Vac/Ch1 group and 96 ± 37.6 in the pigs in the Vac/Ch2 group at 21 d after challenge. The pigs in the Vac/Ch1 group produced significantly higher ($P < 0.05$) numbers of challenging type 1 PRRSV-specific

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Table II. Prevalence of PRRSV viremia, mean lung lesion score, and mean score for number of PRRSV-positive cells per unit area of lung within lesions after challenge, as determined by *in-situ* hybridization for the detection and differentiation of types 1 and 2 PRRSV nucleic acids in the lung tissue

Group and day after challenge	Number of pigs with PRRSV viremia		Score for lung lesions, mean ± standard deviation		Score for number of PRRSV-positive cells, mean ± standard deviation	
	Type 1	Type 2	Macroscopic	Microscopic	Type 1	Type 2
Vac/Ch1						
7	18/18	0/18	52.5 ± 8.8	1.92 ± 2.4	21.7 ± 8.0	0
14	6/12	0/12	26.6 ± 5.1	0.94 ± 0.4[a]	9.3 ± 5.6[a]	0
21	1/6	0/6	5.0 ± 5.4	0.19 ± 0.19[a]	7.4 ± 3.5[a]	0
UnVac/Ch1						
7	18/18	0/18	54.1 ± 10.6	2.19 ± 2.4	23.1 ± 2.0	0
14	10/12	0/12	30.0 ± 8.9	1.63 ± 0.64[a]	18.5 ± 2.7[a]	0
21	5/6	0/6	11.6 ± 18.3	0.63 ± 0.37[a]	12.3 ± 3.1[a]	0
Vac/Ch2						
7	0/18	18/18[b]	6.6 ± 11.6	2.58 ± 0.17[b]	0	39.1 ± 13.2
14	0/12	12/12[b]	53.3 ± 4.0[b]	2.27 ± 0.68	0	29.8 ± 4.2[b]
21	0/6	4/6[b]	20.0 ± 8.9	1.47 ± 0.16[b]	0	18.8 ± 8.0[b]
UnVac/Ch2						
7	0/18	18/18[b]	83.3 ± 2.5	3.08 ± 0.27[b]	0	41.6 ± 8.5
14	0/12	12/12[b]	68.3 ± 11.2[b]	2.83 ± 0.52	0	41.1 ± 8.9[b]
21	0/6	6/6[b]	30.8 ± 8.0	2.47 ± 0.41[b]	0	33.4 ± 13.0[b]
Vac/UnCh						
7	0/18	0/18	0	0	0	0
14	0/12	0/12	2.0 ± 2.5	0.1 ± 0.16	0	0
21	0/6	0/6	3.0 ± 2.8	0	0	0
UnVac/UnCh						
7	0/18	0/18	0	0	0	0
14	0/12	0/12	0	0	0	0
21	0/6	0/6	2.0 ± 3.2	0.2 ± 0.17	0	0

[a] Significant difference ($P < 0.05$) between the Vac/Ch1 and UnVac/Ch1 groups on the same day after challenge.
[b] Significant difference ($P < 0.05$) between the Vac/Ch2 and UnVac/Ch2 groups on the same day after challenge.

IFN-γ-SCs from −14 to 21 d after challenge compared with the pigs in the UnVac/Ch1 group (Figure 5A). Similarly, the pigs in the Vac/Ch2 group produced significantly higher ($P < 0.05$) numbers of challenging type 2 PRRSV-specific IFN-γ-SCs from −14 to 21 d after challenge compared with the pigs in the UnVac/Ch2 group (Figure 5B). The mean number of challenging PRRSV-specific IFN-γ-SCs remained at basal levels (< 20 per 10^6 PBMCs) in the negative-control pigs throughout the experiment.

The pigs in the Vac/Ch1 group had significantly higher ($P < 0.05$) proportions of $CD4^+CD8^+IFN\text{-}\gamma^+$ cells among the PBMCs than the pigs in the UnVac/Ch1, Vac/UnCh, and UnVac/UnCh groups at 21 d after challenge (Figure 6A). Similarly, the pigs in the Vac/Ch2 group had significantly higher ($P < 0.05$) proportions of $CD4^+CD8^+IFN\text{-}\gamma^+$ cells than the pigs in the UnVac/Ch2, Vac/UnCh, and UnVac/UnCh groups at 21 d after challenge (Figure 6B).

Lung lesions were observed in pigs from the challenged groups (Vac/Ch1, UnVac/Ch1, Vac/Ch2, and UnVac/Ch2). Macroscopic lung lesions were mottled or diffusely tan. The affected lungs often failed to collapse, the parenchyma being firmer and heavier than that of the lungs from the negative-control pigs. The mean scores for

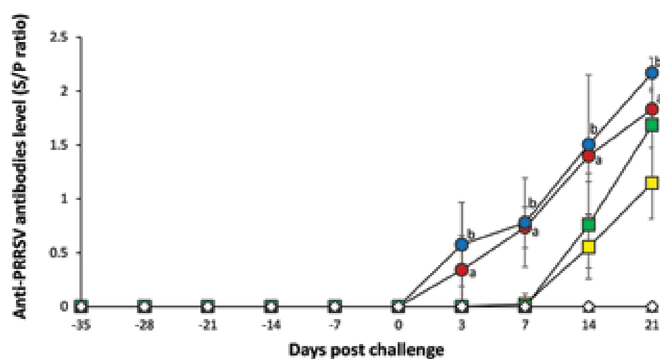

Figure 3. Mean sample/positive (S/P) ratios of anti-PRRSV antibodies in the serum of the pigs. Symbols, variation, and superscript letters (a,b) as for Figure 1.

the macroscopic lung lesions were significantly lower ($P < 0.05$) for the pigs in the Vac/Ch2 group than for the pigs in the UnVac/Ch2 group 14 d after challenge (Table II). Microscopic lung lesions were characterized by interstitial pneumonia with thickened alveolar septa and increased numbers of interstitial macrophages and lymphocytes.

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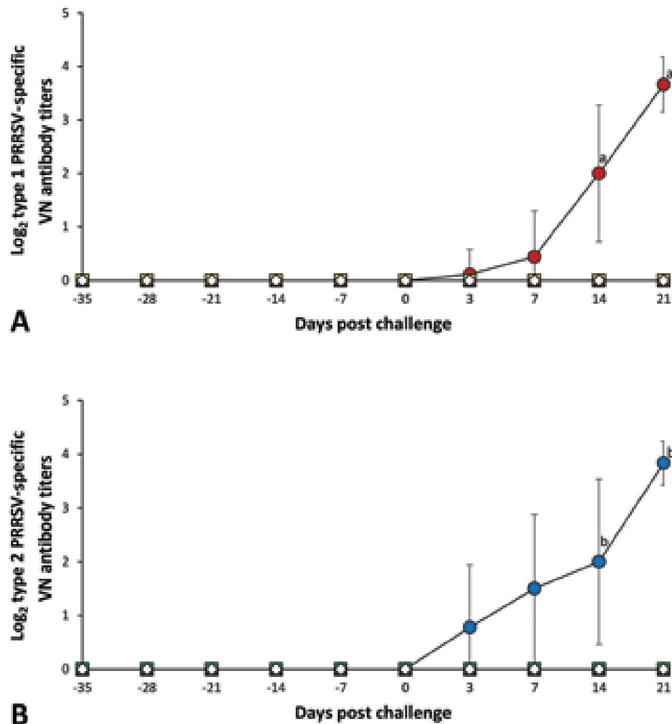

Figure 4. Mean titers of virus-neutralizing (VN) antibody specific for type 1 (A) or type 2 (B) PRRSV in the serum of the pigs. Symbols, variation, and superscript letters (a,b) as for Figure 1.

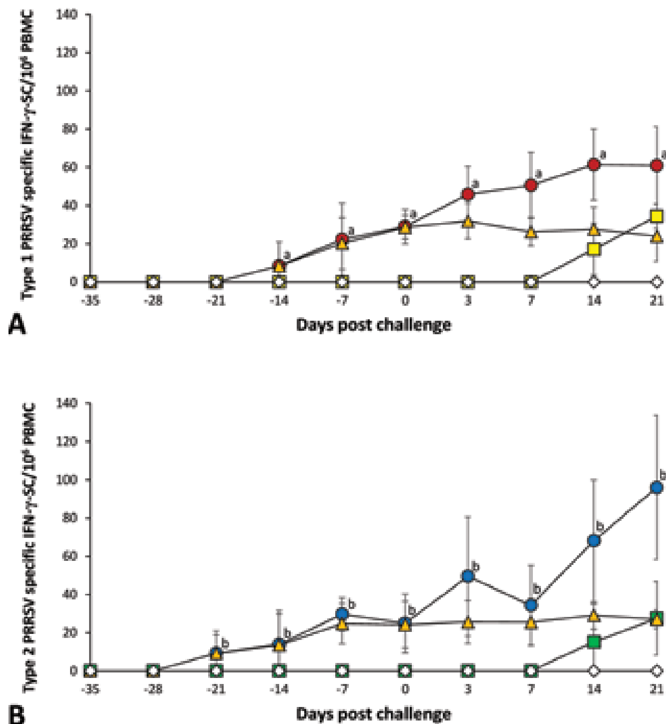

Figure 5. Mean numbers of interferon gamma (IFN-γ)-secreting cells (IFN-γ-SCs) specific for type 1 (A) or type 2 (B) PRRSV among peripheral blood mononuclear cells (PBMCs) from the pigs. Symbols, variation, and superscript letters (a,b) as for Figure 1.

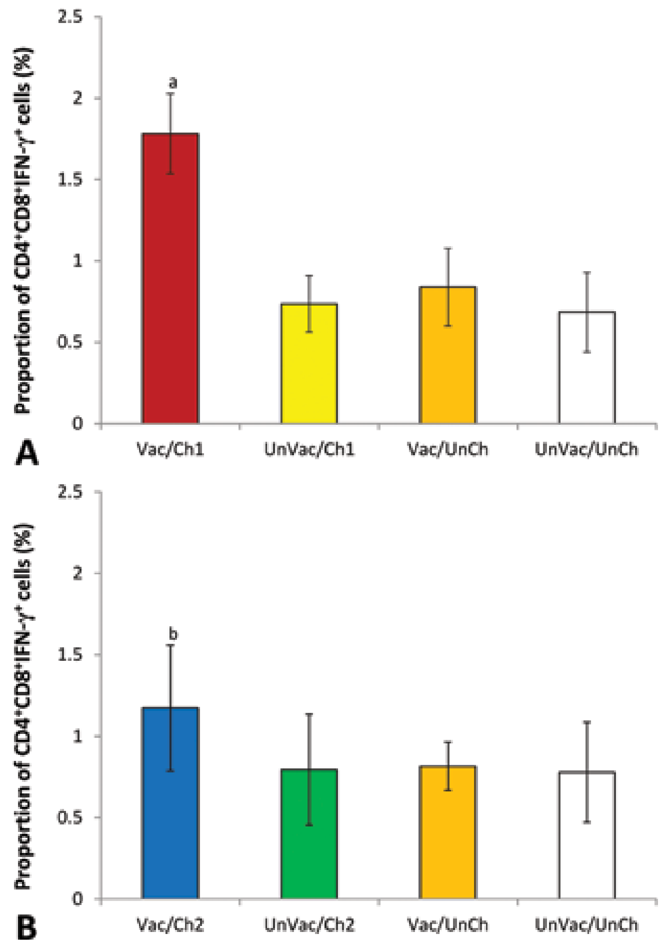

Figure 6. Proportions of $CD4^+CD8^+IFN\text{-}\gamma^+$ cells specific for type 1 (A) or type 2 (B) PRRSV among the PBMCs at 21 d after challenge in the pigs in the Vac/Ch1 (■), UnVac/Ch1 (■), Vac/Ch2 (■), UnVac/Ch2 (■), Vac/UnCh (■), and UnVac/UnCh (□) groups. Variation and superscript letters (a,b) as for Figure 1.

The mean scores for the microscopic lung lesions were significantly lower ($P < 0.05$) for the pigs in the Vac/Ch1 group than for the pigs in the UnVac/Ch1 group 14 and 21 d after challenge. Similarly, the mean scores for the microscopic lung lesions were significantly lower ($P < 0.05$) for the pigs in the Vac/Ch2 group than for the pigs in the UnVac/Ch2 group 7 and 21 d after challenge (Table II). No microscopic or macroscopic lung lesions were detected in the pigs in the Vac/UnCh and UnVac/UnCh groups throughout the experiment.

Nucleic acids from PRRSV were detected exclusively within the cytoplasm of macrophages and pneumocytes. Positive cells typically exhibited a dark brown to black reaction product by *in-situ* hybridization (Figure 7). The mean number of type 1 PRRSV-positive cells per unit area of lung was significantly lower ($P < 0.05$) in the pigs in the Vac/Ch1 group (Figure 7A) than in the pigs in the UnVac/Ch1 group (Figure 7B) at 14 and 21 d after challenge (Table II). Similarly, the mean number of type 2 PRRSV-positive cells per unit area of lung was significantly lower ($P < 0.05$) in the pigs in the Vac/Ch2 group (Figure 7C) than in the pigs in the UnVac/Ch2 group (Figure 7D) at 14 and 21 d after challenge (Table II). Type 1 PRRSV-positive cells were not detected in the lungs of the pigs in the Vac/Ch2

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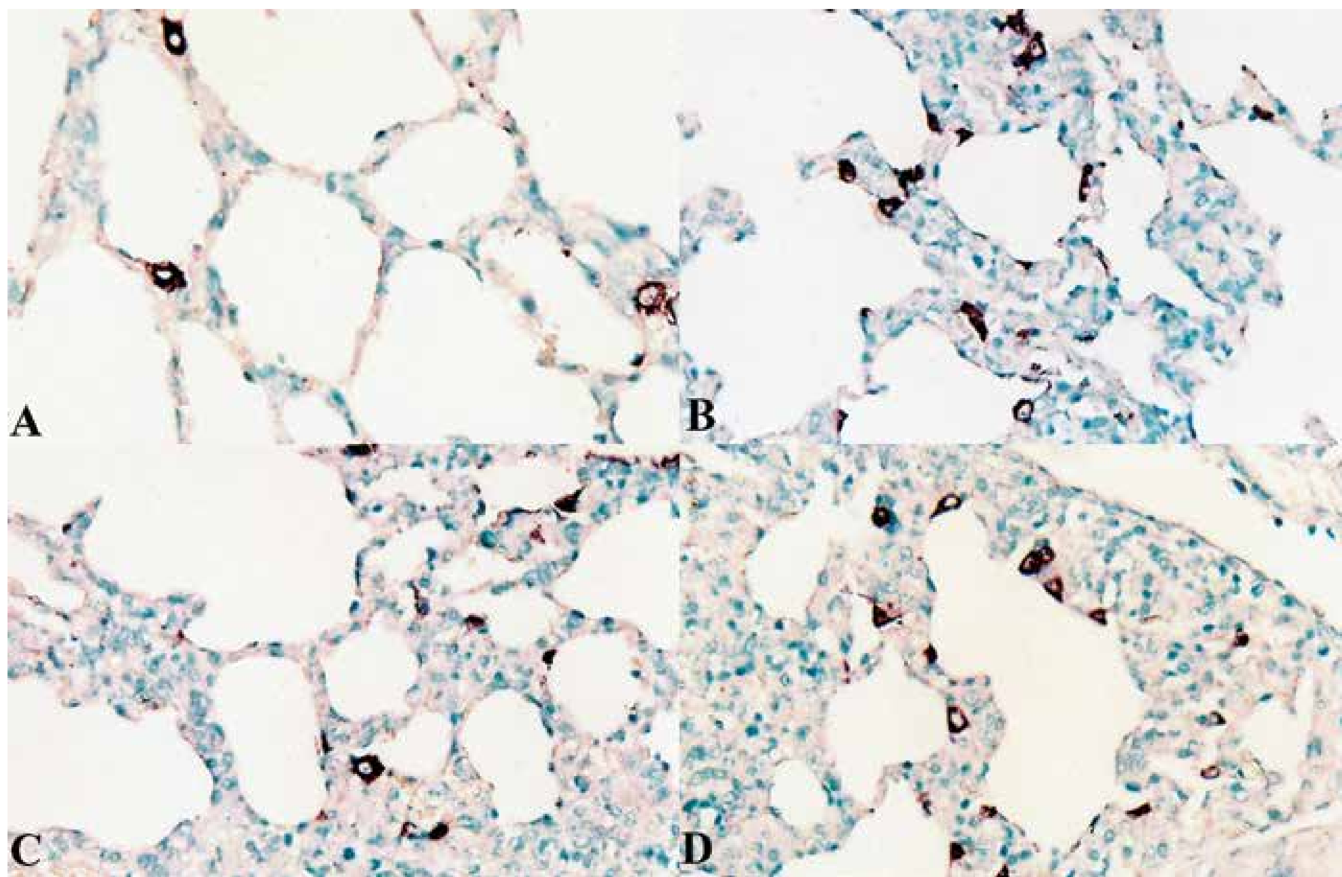

Figure 7. Results of *in-situ* hybridization for the detection of type 1 (A and B) and type 2 (C and D) PRRSV nucleic acids in the lungs of pigs in the Vac/Ch1 (A), UnVac/Ch1 (B), Vac/Ch2 (C), and UnVac/Ch2 (D) groups 14 d after challenge.

and UnVac/Ch2 groups, and vice versa. No PRRSV-positive cells were observed in lung sections from the pigs in the Vac/UnCh and UnVac/UnCh groups.

The type 1 PRRSV isolated by cell culture from pigs in the Vac/Ch1 group (6 pigs at 7 d after challenge, 5 pigs at 14 d, and 3 pigs at 21 d) and the UnVac/Ch1 group (6 pigs at 7 and 14 d and 4 pigs at 21 d) was confirmed by sequencing to be identical to the type 1 PRRSV in the challenge stock; no type 2 PRRSV was isolated from pigs in these 2 groups. The type 2 PRRSV isolated by cell culture from pigs in the Vac/Ch2 group (6 pigs at 7 d after challenge, 5 pigs at 14 d, and 3 pigs at 21 d) and the UnVac/Ch2 group (6 pigs at 7, 14, and 21 d) was confirmed by sequencing to be identical to the type 2 PRRSV in the challenge stock; no type 1 PRRSV was isolated from pigs in these 2 groups. No type 1 or type 2 PRRSV was isolated from the pigs in the unchallenged groups.

Discussion

The results of the present study demonstrate that the new commercial recombinant chimeric PRRS vaccine provides protection against challenge with heterologous types 1 and 2 PRRSV. Vaccination of pigs with this new vaccine results in reduction of viremia levels, lung lesions, and types 1 and 2 PRRSV antigens within lung lesions from those observed in pigs challenged but not vaccinated. Our results agree with previous findings that vaccination of pigs with recombinant vaccines resulted in partial protection, with reduction in type 1 and type 2 PRRSV viremia and lung lesions (36,37). In contrast, respiratory disease was exacerbated in pigs challenged with PRRSV after vaccination with a recombinant GP5 subunit PRRSV vaccine produced in *Escherichia coli* (38). We have no clear explanation about the conflicting results, but they may be due to differences in viral glycoprotein and adjuvant between the 2 vaccines. The latter vaccine contains only GP5, which is encoded by ORF5, contains the main neutralizing epitope (22,39,40) of both type 1 (41,42) and type 2 (43,44) PRRSV isolates, and is responsible for immunodominant T-cell epitopes that stimulate IFN-γ-SCs (45). Vaccination with proteins GP5 and M of PRRSV confers a certain degree of protection (36,39,40). In a previous study, mice vaccinated with a detoxified *Pseudomonas* exotoxin-based human papillomavirus subunit vaccine exhibited a significant increase in the numbers of virus-specific $CD4^+$ and $CD8^+$ T-cells compared with mice treated with the subunit vaccine alone (46). Similarly, in the present study the recombinant chimeric PRRS vaccine increased the number of $CD4^+CD8^+IFN\text{-}\gamma^+$ cells, which constitute the majority of IFN-γ-SCs (33). Therefore, it is possible that the *Pseudomonas* exotoxin plays an important role in enhancing the cellular immune response. Likewise, pigs vaccinated with a killed virus have been shown to have $CD4^+CD8^+$ T-cells that were preferentially recalled upon exposure to the live virus, which suggested the presence of virus-specific memory cell pools (47).

Selection of an appropriate challenge virus is critical to evaluating the efficacy of a vaccine. The challenge strains of types 1 and 2

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PRRSV are virulent, causing interstitial pneumonia. In particular, the type 1 challenge virus used in this study is more pathogenic than any other Korean type 1 PRRSV isolates examined (25,26,48). In addition, several peer-reviewed publications describing the efficacy of PRRS vaccine reported using the same challenge strains (16,30,49,50). The recombinant chimeric PRRS vaccine augments anamnestic virus-specific VN and IFN-γ responses after a wild-type virus challenge, which contributes to viral clearance, as happens with inactivated PRRS vaccines (32,47,51).Vaccination of pigs with the recombinant chimeric PRRS vaccine is not able to attenuate peak viremia at 7 d after challenge but thereafter reduces the levels of types 1 and 2 PRRSV viremia consistently up to 21 d after challenge. In spite of the variable VN antibody titers and numbers of IFN-γ-SCs in vaccinated animals, viral clearance from the blood coincides with the induction of VN antibodies and IFN-γ-SCs.

The cocirculation of PRRS viruses of both genotypes, 1 and 2, is an important clinical issue in some parts of the world, most notably in East Asia, including Korea. The protection afforded by the recombinant chimeric PRRS vaccine against types 1 and 2 PRRSV is clinically significant because a commercial divalent vaccine against types 1 and 2 PRRSV is not yet available. Moreover, concurrent vaccination of pigs against types 1 and 2 PRRSV protects growing pigs against respiratory diseases caused by type 1 PRRSV but not against those caused by type 2 PRRSV (50). From the aspect of safety, inactivated PRRS vaccines are preferred over live-attenuated vaccines because a live-attenuated vaccine virus has been shown to revert to virulence under field conditions (52). The results of the present study demonstrate that, with the use of an optimized selection of PRRSV subunits in combination with a *Pseudomonas* exotoxin carrier, a recombinant chimeric PRRS vaccine can induce VN antibodies and IFN-γ-SCs and offer protection upon heterologous types 1 and 2 PRRSV challenge. Thus, this novel recombinant chimeric PRRS vaccine is a promising alternative to control both type 1 and type 2 PRRSV infection effectively and safely.

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Analysis of efficacy obtained with a trivalent inactivated *Haemophilus parasuis* serovars 4, 5, and 12 vaccine and commercial vaccines against Glässer's disease in piglets

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Abstract

The objective of this study was to assess the efficacy of a trivalent inactivated *Haemophilus parasuis* serovars 4, 5, and 12 vaccine with polymeric adjuvant gel (GEL) and commercial vaccines against Glässer's disease in piglets. Commercial vaccines containing inactivated *H. parasuis* serovars 4 and 5 (China), inactivated *H. parasuis* serovars 1 and 6 (Spain), and inactivated *H. parasuis* serovar 5 (USA) were also evaluated. Our results demonstrated that the trivalent inactivated *H. parasuis* serovars 4, 5, and 12 vaccine with GEL adjuvant can provide better protection against the 3 most common pathogenic serovars circulating in China than other commercial vaccines tested. Our findings also indicated that inactivated *H. parasuis* serovars 1 and 6 vaccine cross-protects piglets against *H. parasuis* serovars 4 and 5; inactivated *H. parasuis* serovar 5 vaccine cross-protects piglets against *H. parasuis* serovar 4 challenge; but none of the commercial vaccines tested in this study protected piglets against *H. parasuis* serovar 12. Our results provide a basis for further identification of common protective antigens that can induce cross-protection against heterogeneous serovars.

Résumé

L'objectif de la présente étude était d'évaluer l'efficacité d'un vaccin inactivé trivalent contre Haemophilus parasuis *contenant les sérovars 4, 5 et 12 avec un adjuvant en gel polymérique (GEL) et des vaccins commerciaux contre la maladie de Glässer chez des porcelets. Des vaccins commerciaux contenant du* H. parasuis *inactivé sérovars 4 et 5 (Chine), du* H. parasuis *inactivé sérovars 1 et 6 (Espagne), et du* H. parasuis *inactivé sérovar 5 (États-Unis) ont également été évalués. Nos résultats démontrent que le vaccin inactivé trivalent contenant* H. parasuis *sérovars 4, 5 et 12 avec l'adjuvant GEL peut fournir une meilleure protection contre les trois sérovars les plus fréquents circulant en Chine que les autres vaccins commerciaux testés. Nos trouvailles indiquent également que le vaccin contenant* H. parasuis *inactivé sérovars 1 et 6 offre une protection croisée contre* H. parasuis *sérovars 4 et 5; le vaccin inactivé* H. parasuis *sérovar 5 offre une protection croisée contre un challenge avec* H. parasuis *sérovar 4; mais qu'aucun des vaccins commerciaux testés dans l'étude ne protège les porcelets contre* H. parasuis *sérovar 12. Nos résultats fournissent une base pour l'identification future d'antigènes protecteurs communs qui peuvent induire une protection contre des sérovars hétérologues.*

(Traduit par Docteur Serge Messier)

Introduction

Haemophilus parasuis is a Gram-negative, nicotinamide adenine dinucleotide (NAD)-dependent strain that is the causative agent of Glässer's disease, which is characterized by polyserositis, meningitis, and arthritis in swine (1–3). *Haemophilus parasuis* primarily causes disease in 4- to 12-week-old piglets and results in significant losses in the swine industry (4,5). To date, 15 serovars of *H. parasuis* have been described, but some isolates are not typable (1). Numerous field isolates have been identified and serovars 4, 5, 14, 13, and 12 are considered the most prevalent *H. parasuis* strains in China (6–8).

Vaccination is thought by some to be the most effective means of controlling Glässer's disease (9). Subunit vaccines containing recombinant transferrin-binding protein B (TbpB); outer membrane protein (OMP) formulations enriched with TbpB; and OMP2 or D15, PalA, and HPS-06257 from OMP provide partial protection against *H. parasuis* challenge (9–11). Ghost vaccines are also promising (10). These vaccines usually give protection against challenge with the heterologous serotype, but few studies of cross-protection have been reported (12). Currently, an inactivated *H. parasuis* serovar 4 and 5 vaccine, a serovar 5 vaccine, and a serovar 1 and 6 vaccine against Glässer's disease are the primary commercial vaccines used in China, the USA, and Spain, respectively (13). These vaccines play an important role in preventing and controlling Glässer's disease.

In previous studies, we screened strongly immunogenic isolates of serovars 4, 5, and 12 as candidate vaccine strains (14), and concluded that polymeric adjuvant gel (GEL) should be used as a candidate adjuvant of a trivalent inactivated *H. parasuis* serovars 4, 5, and 12 vaccine (15). In the present study, we assessed the safety, immune response, and protective efficacy of trivalent inactivated *H. parasuis*

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serovars 4, 5, and 12 vaccine with GEL adjuvant and 3 commercially available vaccines against Glässer's disease in piglets.

Materials and methods

Haemophilus parasuis isolates

Haemophilus parasuis serovar 4, 5, and 12 isolates, which were isolated and typed by our laboratory, were used for vaccine production. *Haemophilus parasuis* was cultured on tryptic soy agar supplemented with 10 μg/mL NAD and 5% fetal calf serum and incubated at 37°C for 48 h. The isolates were suspended in 15% skimmed milk and stored at −80°C until further use.

Adjuvants

Polymeric gel adjuvant (Montanide GEL 01 PR; SEPPIC, Paris, France) was used. This is a new adjuvant based on the dispersion of a high molecular weight polyacrylic polymer in water.

Vaccine formulations

Four vaccines (F1–F4) were used in this study. The F1 vaccine contained GEL and 3 inactivated Chinese field isolates of *H. parasuis* serovars 4, 5, and 12 at concentrations of 2.0×10^9, 1.0×10^9, and 1.0×10^9 CFU/mL, respectively. This vaccine was tested for physical properties and sterility as described by the veterinary biological products procedures of China. The F2 vaccine (with mineral oil adjuvant) is a commercially available bacterin (China Animal Husbandry Industry Company, Beijing, China) that contains inactivated *H. parasuis* serovars 4 and 5 at concentrations of 4.0×10^9 and 4.0×10^9 CFU/mL, respectively. The F3 vaccine is a commercially available bacterin (Laboratorios HIPRA S.A., Amer, Spain) that contains inactivated *H. parasuis* serovars 1 and 6 at concentrations of 2.0×10^9 and 2.0×10^9 CFU/mL, respectively. The F4 vaccine is a commercially available bacterin (Boehringer Ingelheim, Columbus, Ohio, USA) that contains inactivated *H. parasuis* serovar 5 at a concentration of 1.5×10^9 CFU/mL.

Animals

Female Landrace × large white piglets aged 3 to 4 wk were purchased (Shengping, Luoyang, China). A single nasal swab obtained from each piglet was used for polymerase chain reaction (PCR) to test for negative status (16) to *H. parasuis*. The piglets were serologically negative by microagglutination test, as described previously (15).

Vaccine safety

Twenty-five piglets were randomly assigned to 5 groups (A1–A5) of 5 animals each and housed in isolation in pens with a concrete floor and an automatic watering system. Groups A1–A4 were vaccinated intramuscularly in the neck with 4 mL per piglet of the F1–F4 vaccines, respectively. Group A5 was injected with 4 mL of sterile phosphate buffered saline (PBS) as a control. Every day, from 3 d before experimental inoculation until the first 7 d post-vaccination, rectal temperature was measured. The threshold of fever was established at 39.5°C and animals with rectal temperature > 39.5°C were considered febrile. To evaluate the safety of these vaccines, we developed a method of safety assessment according to a scoring system based on clinical signs and adverse reactions caused by vaccines (Table I), including depression, anorexia, fever, diarrhea, emesis, granulation tissue hyperplasia, and vaccine residue at the injection site, as described previously (17). All the animals were subjected to necropsy. Gross lesions were recorded with special attention to reaction at the injection site; the pleural, pericardial and peritoneal cavities; the hock, carpal, and stifle joints; lungs; and central nervous system.

Immune responses and protective efficacy

Seventy-five piglets were randomly assigned to 15 groups (E1–E15) of 5 animals each and housed in isolation in pens with a concrete floor. Food and water were provided *ad libitum* throughout the study. Groups E1–E3, E4–E6, E7–E9, and E10–E12 were vaccinated intramuscularly in the neck with 2 mL per piglet of F1–F4 vaccines, respectively, and given similar booster injections 21 d later. Groups E13–E15 were injected with 2 mL sterile PBS as a control on the same day that other groups were vaccinated. Fourteen days after the second immunization, groups E1, E4, E7, E10, and E13 were challenged intraperitoneally (IP) with 9.0×10^9 CFU (18) of *H. parasuis* serovar 4; groups E2, E5, E8, E11, and E14 were challenged IP with 5.2×10^9 CFU (18) of *H. parasuis* serovar 5; and groups E3, E6, E9, E12, and E15 were challenged IP with 3.5×10^9 CFU (18) of *H. parasuis* serovar 12. The experiment was terminated 15 d post-challenge. At the end of the experiment, survivors were euthanized using an intravenous overdose of sodium pentobarbital. All animal handling procedures were approved by the Biosafety and Animal Welfare Committee of the institute. All the animals were subjected to necropsy and any gross lesions of the thoracic cavity and peritoneal cavities were recorded. Samples were obtained for bacterial isolation.

Blood samples from piglets were collected by venipuncture from the jugular vein before each immunization (days 0 and 21), before challenge (day 35), and 5 d after challenge (day 40). Serum was obtained and stored at −80°C prior to microagglutination test analysis (15).

Statistical analysis

Statistical analysis to assess significant differences in rectal temperature before and after vaccination was done using the *t*-test, and analysis of antibody titers were done using a one-way analysis of variance (ANOVA). A $P < 0.05$ was considered significant. The analysis was conducted using computer software (SPSS Statistics for Windows, version 17.0; SPSS, Chicago, Illinois, USA).

Results

Safety

No clinical signs or pathological findings were observed in any piglets injected with sterile PBS (Group A5; Table II). With the exception of fever, no clinical signs were observed in any of the vaccinated animals. Mean rectal temperature in the vaccinated groups (A1–A4) showed an increase (39.5°C to 40.3°C) 24 h after vaccination, which was significant compared to the rectal temperature before inoculation ($P < 0.05$). However, all of the piglets returned to normal body

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Table I. Score used for evaluating safety of vaccines

Category of clinical sign	Range	Score			
		Normal	Slight	Moderate	Severe
Systemic	Depression	0	1	2	3
	Anorexia	0	1	2	3
	Febrile	0	1	2	3
	Diarrhea	0	1	2	3
	Emesis	0	1	2	3
Injection site	Granulation tissue hyperplasia	0	1	2	3
	Vaccine residue	0	1	2	3

Table II. Vaccine safety assessment on piglets

Vaccine type	Number of piglets	Depression	Anorexia	Febrile	Diarrhea	Emesis	Granulation tissue hyperplasia	Vaccine residue	Score
F1	1	0	0	1	0	0	0	0	5
	2	0	0	1	0	0	0	0	
	3	0	0	1	0	0	0	0	
	4	0	0	1	0	0	0	0	
	5	0	0	1	0	0	0	0	
F2	1	0	0	1	0	0	3	3	35
	2	0	0	1	0	0	3	3	
	3	0	0	1	0	0	3	3	
	4	0	0	1	0	0	3	3	
	5	0	0	1	0	0	3	3	
F3	1	0	0	1	0	0	0	0	5
	2	0	0	1	0	0	0	0	
	3	0	0	1	0	0	0	0	
	4	0	0	1	0	0	0	0	
	5	0	0	1	0	0	0	0	
F4	1	0	0	1	0	0	0	0	5
	2	0	0	1	0	0	0	0	
	3	0	0	1	0	0	0	0	
	4	0	0	1	0	0	0	0	
	5	0	0	1	0	0	0	0	
Control	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	
	5	0	0	0	0	0	0	0	

0 — Normal; 1 — Slight; 2 — Moderate; 3 — Severe.

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Table III. Mean antibody titer of 5 groups of piglets for 4 vaccines

Vaccine types[a]	Before first immunization (day 0)			Before second immunization (day 21)			Before challenge (day 35)			After challenge (day 40)		
(Groups)	4	5	12	4	5	12	4	5	12	4	5	12
F1 (E1–E3)	0	0	0	1:8	1:12	1:16	1:28	1:32	1:37	1:64	1:64	1:128
F2 (E4–E6)	0	0	0	1:12	1:16	0	1:24	1:24	0	1:32	1:64	0
F3 (E7–E9)	0	0	0	1:8	1:8	0	1:16	1:12	0	1:32	1:24	0
F4 (E10–E12)	0	0	0	0	1:12	0	1:8	1:24	0	1:16	1:64	0
Control (E13–15)	0	0	0	NT	NT	NT	NT	NT	NT	NT	NT	NT

[a] F1 vaccine — an inactivated vaccine that contains *H. parasuis* serovars 4, 5, and 12; this study. F2 vaccine — a commercially available bacterin that contains inactivated *H. parasuis* serovars 4 and 5; China. F3 vaccine — a commercially available bacterin that contains inactivated *H. parasuis* serovars 1 and 6; Spain. F4 vaccine — a commercially available bacterin that contains inactivated *H. parasuis* serovars 5; USA.
NT — not tested.
The antibody titers of the serum were expressed as the reciprocal of the dilution, which neutralized 50% of the antigen. 4, 5, 12: *H. parasuis* serovars 4, 5, and 12, respectively. The antibody titers in F1 vaccine group were significant ($P < 0.05$) compared to F2, F3, and F4 vaccine groups.

Table IV. Protective efficacy of the 4 vaccines

Vaccine types	Number of piglets	Vaccination	Dose (mL)	*H. parasuis* and protective efficacy: Serovars 4	Serovars 5	Serovars 12
F1	15	Intraperitoneal	2	100%	100%	100%
F2	15	Intraperitoneal	2	100%	100%	0%
F3	15	Intraperitoneal	2	100%	80%	0%
F4	15	Intraperitoneal	2	60%	100%	0%
Control	15	Intraperitoneal	2	0%	0%	0%

temperature within 48 h after vaccination. Piglets in group A2 showed signs of vaccine residue and granulation tissue hyperplasia at the injection site. In total, the adverse reaction scores associated with the vaccines ranged from small to large, as follows: F1 (score 5), F3 (score 5), F4 (score 5), and F2 (score 35).

Analysis of antibody titers

The antibody titers of groups E1 to E15 are presented in Table III. No antibody response was found in the control group (F5) before challenge. Antibody response after challenge could not be measured in this group because all pigs died within 3 d of challenge. *Haemophilus parasuis* serovars 4 and 5 antibodies were detected in all immunized groups (E1–E12) following primary and secondary immunization, and increased over time. The antibody titers in the F1 vaccine group (E1–E3) were significantly greater ($P < 0.05$) compared with those in the F2–F4 vaccine groups, because the F1 vaccine elicited antibodies to *H. parasuis* serovars 4, 5, and 12, but no antibodies to *H. parasuis* serovar 12 were observed in the F2–F4 vaccine groups. The F3 vaccine elicited antibodies to *H. parasuis* serovars 4 and 5, although this vaccine only consisted of inactivated *H. parasuis* serovars 1 and 6. Similarly, F4 vaccine elicited antibodies to *H. parasuis* serovar 4, although this vaccine only contained inactivated *H. parasuis* serovar 5.

Protective efficacy of vaccines

Protective efficacy of the vaccines is presented in Table IV. All the piglets injected with sterile PBS (groups E13–E15) developed signs of Glässer's disease, such as serofibrinous exudates, polyserositis of varying severity within the pericardium, thoracic cavity, and enterocelia, and began to die at 8 h post-challenge and all were dead by 3 d post-challenge. The F1 vaccine provided 100% protection against IP challenge with *H. parasuis* serovars 4, 5, and 12. The F2 vaccine provided 100% protection against *H. parasuis* serovars 4 and 5, but zero protection against serovar 12. The F3 vaccine provided 100% and 80% protection against *H. parasuis* serovars 4 and 5, respectively, although this vaccine only contained inactivated *H. parasuis* serovars 1 and 6. The F4 vaccine provided 100% protection against *H. parasuis* serovar 5, but only 60% protection against serovar 4 and zero protection against serovar 12. *Haemophilus parasuis* was isolated from tissues and organs of all dead piglets, which exhibited similar symptoms and pathological changes as in the control groups. However, at the end of the experiment, none of the surviving piglets exhibited symptoms and pathological changes related to Glässer's disease in the upper respiratory tract, as were seen in the control group.

Discussion

Glässer's disease can be severe with high morbidity and mortality rates reported in swine in China and some European countries. To date, effective commercially available vaccines that provide cross-protection against all pathogenic serovars are not available (19,20). An inactivated *H. parasuis* serovars 4 and 5 vaccine is reported to reduce mortality and clinical symptoms in pigs after challenge

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with serovars 4, 5, 13, and 14 (prevalent in China) (13). However, this inactivated *H. parasuis* serovar 4 and 5 vaccine fails to protect pigs from infection with *H. parasuis* serovar 12, which is prevalent in China. Therefore, development of a commercial trivalent inactivated *H. parasuis* serovars 4, 5, and 12 vaccine against Glässer's disease is necessary. Developing a new vaccine model requires convincing data supporting the safety and efficacy of the product. Therefore, safety, immune responses, and protective efficacy of the trivalent inactivated *H. parasuis* serovars 4, 5, and 12 vaccine with GEL adjuvant and commercial vaccines against Glässer's disease was assessed in this study.

The safety experiment indicated that piglets which received F2 vaccine had vaccine residue and granulation tissue hyperplasia at the injection site. Piglets which received F1 vaccine, F3 vaccine, and F4 vaccine had no vaccine residue and granulation tissue hyperplasia at the injection site. These results implied that the F1, F3, and F4 vaccines were safer than the F2 vaccine, which probably resulted from different adjuvant formulations.

The antibody responses and protective efficacy experiment indicated that the F2, F3, and F4 vaccines cannot protect against challenge with *H. parasuis* serovar 12, while the F1 vaccine elicited a measurable antibody response and protected against challenge with *H. parasuis* serovars 4, 5, and 12. This suggests that F1 vaccine provides protection against 3 of the common pathogenic serovars circulating in China. Field trials of the trivalent, inactivated *H. parasuis* serovars 4, 5, and 12 vaccine administered with GEL should be conducted on pig farms in the future.

Nielsen (21) reported that pigs given pathogenic serovars 2, 3, 4, or 7 strains of *H. parasuis* via aerosol can resist challenge with serovar 5. Takahashi et al (22) were unable to demonstrate cross-protection with a serovar 2 and 5 bacterin. Brockmeier et al (4) reported that inactivated SW113 vaccine and live SW113 vaccine (serovar 3) protected against challenge with heterologous strain 12939 (serovar 4) of *H. parasuis.* Huang Xiaohui et al (19) reported that transferrin-binding protein A (TbpA) cloned from LJ3 (serovar 13) might function as a new protective antigen, which protects against challenge with homologous LJ3 (serovar 13) and heterologous FX1 (serovar 4), and SZ (serovar 14) strains. In the present study, F3 vaccine (containing *H. parasuis* serovars 1 and 6) elicited an antibody titer to *H. parasuis* serovars 4 and 5 and provided 100% and 80% protective efficacy against *H. parasuis* serovars 4 and 5, respectively. This is believed to be the first study to suggest that *H. parasuis* serovars 1 and 6 and serovars 4 and 5 have common protective antigens. The likely common protective antigen, such as OMP or transferrin-binding protein, should be identified in future studies. The F4 vaccine (containing *H. parasuis* serovar 5) elicited antibody titer to *H. parasuis* serovar 4 and provided 60% protective efficacy against *H. parasuis* serovars 4. This indicated that *H. parasuis* serovars 4 and 5 have common protective antigens, which is similar to the study by Nielsen (21). However, the specific common protective antigens that may be predicted by bioinformatics analysis between *H. parasuis* serovars 4 and 5 are still unclear and should be identified in future studies. Additionally, F2–F4 vaccines did not cross-protect pigles against *H. parasuis* serovar 12, suggesting that *H. parasuis* serovars 1, 4, 5, 6, and 12 may have no common protective antigens.

Our results demonstrated that a trivalent inactivated *H. parasuis* serovars 4, 5, and 12 vaccine with GEL can provide better protection against 3 pathogenic serovars circulating in China than any other commercial vaccine tested. Our findings also provide a basis for further identification of common protective antigens that can induce cross-protection against heterologous serovars.

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Article

Phylogenetic analysis of *Escherichia coli* isolated from broilers with colibacillosis based on *gyrA* gene sequences

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Abstract

Escherichia coli isolates from chickens with colibacillosis were assigned to phylogenetic groups based on multiplex polymerase chain reaction (PCR) and antibacterial resistance of *E. coli* belonging to these groups was examined. Furthermore, the *gyr*A gene of isolates was sequenced and a phylogenetic tree was generated. A total of 84 *E. coli* isolates were grouped using multiplex PCR of TSPE4.C2, *chu*A, *yja*A, and *gad*A molecular markers. Four phylogenetic groups were identified with strains divided as follows: 16 in group A (19.05%), 17 in group B1 (20.24%), 23 in group B2 (27.38%), and 28 in group D (33.33%). *Escherichia coli* isolates belonging to phylogenetic groups B2 and D were resistant to Soltrim and Flumequine unlike the majority of *E. coli* isolates that belonged to groups A and B1, and which were susceptible to these antibiotics. The phylogenetic results based on *gyrA* gene sequences from multiplex PCR revealed that *E. coli* phylogenetic grouping was in accordance with the clusters obtained in the phylogenetic tree. In conclusion, the comparative sequence analysis of *gyrA* sequences provides a firm framework for an accurate classification of *E. coli* and related taxa and may constitute a pertinent phylogenetic marker for *E. coli.*

Résumé

Les isolats d'Escherichia coli *provenant de poulets avec colibacillose ont été assignés à des groupes phylogénétiques sur la base d'une réaction d'amplification en chaine par la polymérase multiplex (ACP) et la résistance antimicrobienne des* E. coli *appartenant à ces groupes a été examinée. De plus, le gène* gyr*A des isolats a été séquencé et un arbre phylogénétique a été généré. Un total de 84 isolats a été groupé à l'aide de l'ACP multiplex utilisant les marqueurs moléculaires TSPE4.C2,* chu*A,* yja*A et* gad*A. Quatre groupes phylogénétiques ont été identifiés et les souches réparties comme suit : 16 dans le groupe A (19,05 %), 17 dans le groupe B1 (20,24 %), 23 dans le groupe B2 (27,38 %), et 28 dans le groupe D (33,33 %). Les isolats d'*E. coli *appartenant aux groups phylogénétiques B2 et D étaient résistants au Soltrim et à la fluméquine contrairement à la majorité des isolats d'*E. coli *appartenant aux groupes A et B1, qui étaient sensibles à ces antibiotiques. Les résultats phylogénétiques basés sur les séquences du gène* gyr*A provenant de l'ACP multiplex ont révélé que le regroupement phylogénétique des* E. coli *était en accord avec les groupes obtenus dans l'arbre phylogénétique. En conclusion, l'analyse comparative des séquences* gyr*A fourni un patron solide pour une classification précise des* E. coli *et taxons associés et pourrait constituer un marqueur phylogénétique pertinent pour les isolats d'*E. coli.

(Traduit par Docteur Serge Messier)

Introduction

Avian colibacillosis is caused by avian-pathogenic *Escherichia coli* (APEC), which is the etiological agent of extraintestinal infections including respiratory infections, pericarditis, and septicemia in poultry (1). Avian colibacillosis is the most common infectious bacterial disease in poultry and is responsible for major economic loss in the poultry industry worldwide (2).

Molecular characterization of infectious agents is useful for epidemiological surveillance and public health activities. Based on an improved multiplex polymerase chain reaction (PCR) using the genetic markers *chuA*, *yjaA*, and *gadA*, and the DNA fragment TspE4.C2, *E. coli* strains have been phylogenetically classified into 4 main groups: A, B1, B2, and D (3,4). It has been reported that strains from these 4 groups vary in terms of their characteristics, including antibiotic-resistance level (5) and virulence. Virulent extraintestinal strains mainly belong to group B2 and to a lesser extent, group D (6,7).

Multiplex PCR using genetic markers *chuA*, *yjaA*, *gadA*, and TspE4. C2, is an effective tool for the speciation of *E. coli* isolated from different sources and many researchers have successfully applied this method (3,4,8). However, a high rate of incorrect classifications using multiplex PCR protocol has been reported in previous work (4,9).

DNA gyrase enzyme is composed of 2 subunits, *gyrA* and *gyrB*, which introduce negative supercoils into DNA. This essential bacterial enzyme is found in all bacteria and is conserved among species, making it an attractive target for phylogenetic studies (10). The presence of highly conserved motifs in *gyrA* gene sequences provides a useful tool when designing universal primers for the study of bacterial identity and diversity (11).

Menard et al (12), showed that a phylogenetic tree generated based on *gyrA* gene sequencing enabled reliable clustering of *Helicobacter cinaedi* and *Flexispira* strains. In the present study, a 1.8 kb fragment of the *gyrA* gene from bacterial strains isolated from clinical specimens of poultry with collibacilosis was amplified. This region was

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Table I. Names and accession number of *E. coli* strains used for generating phylogenetic tree

	E. coli strain	Accession number
1	94-3024	CP009106.2
2	E24377A	CP000800.1
3	ECC-1470	NZ_CP010344.1
4	O26:H11 str. 11368	NC_013361.1
5	APEC O78	NC_020163.1
6	ACN001	NZ_CP007442.1
7	BW2952	NC_012759.1
8	ER2796	NZ_CP009644.1
9	P12b	NC_017663.1
10	KLY	NZ_CP008801.1
11	O157:H7 str. SS52	NZ_CP010304.1
12	SMS-3-5	NC_010498.1
13	IAI39	CU928164.2
14	UMN026	CU928163.2
15	NA114	NC_017644.1
16	CFT073	NC_004431.1
17	ED1a	CU928162
18	BL21 (DE3)	CP001665.1
19	B str. REL606	CP000819.1

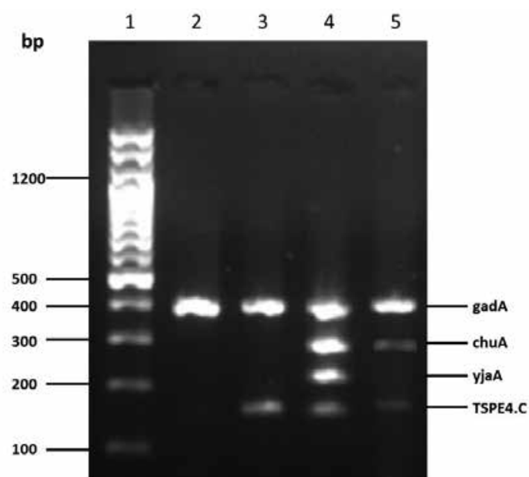

Figure 1. Multiplex PCR profiles showing phylogenetic groups of *E. coli* examined in the present study. Lanes 1 — 100bp molecular marker (Bioflux, South Korea). Lane 2 — Phylogenetic group A. Lane 3 — Phylogenetic group B1. Lane 4 — Phylogenetic group B2. Lane 5 — Phylogenetic group D.

sequenced and the results were used to compile phylogenetic relationships for the isolated bacteria. We presented a new phylogenetic analysis of *E. coli* based on the *gyrA* gene region and compared the results with those of the phylogenetic analysis by multiplex PCR.

Materials and methods

Bacterial isolates

Eighty-four *E. coli* isolates obtained from broiler chickens with colibacillosis from private veterinary laboratories across northern Iran between 2014 and 2015 were used herein. All isolates were identified as *E. coli* by their morphology, growth characteristics, Gram staining, and biochemical methods [indole, methyl red, Voges-Proskauer, citrate (IMViC) tests and urease production, H_2S production, and various sugar fermentation tests] as described by Nolan et al (1).

DNA extraction and multiplex PCR

Bacterial genomic DNA was extracted using the rapid boiling method described by Wang et al (13). A single colony of grown *E. coli* isolate was boiled at 100°C for 5 min in 100 mL of distilled water. After cooling to room temperature, the suspension was centrifuged for 3 min at maximum speed to remove cell debris. Assignment of *E. coli* isolates into phylogenetic groups was performed using the multiplex PCR procedure described by Doumith et al (3).

Antimicrobial susceptibility test

An antibiotic sensitivity test was performed using the Kirby-Bauer method on Müeller-Hinton agar (disc diffusion technique) as described by the Clinical Laboratory Standards Institute (CLSI) (14). Antimicrobial disks are used frequently in the poultry industry to detect antibiotic sensitivity in chickens diagnosed with colibacillosis. Antimicrobial susceptibility of *E. coli* isolates to the following antibiotics was tested: Cefazolin (KZ; 30 μg), Imipenem (IPM; 10 μg), Trimetoprim-sulfametoxazol (SXT; 23.1 + 75.25 μg), Doxycycline (DO; 30 μg), Colistin (CT; 10 μg), Soltrim (ST; 23.75 μg), Flumequine (FLM; 30 μg), Linco-spectin (LP; 15 + 200 μg), Florfenicol (FFC; 30 μg), and Enrofloxacin (ENR; 5 μg).

Amplification of *gyrA* gene

A pair of primers [*gyrA*-F (5′-TTACGGCCCACGATGCTGATTT-3′) and *gyrA*-R (5′-CATCAACGGTTGTCTGGCGTAT-3′)] was designed based on *E. coli gyrA* nucleotide sequences retrieved from GenBank. These primers were used to amplify a 1.8 bp fragment of *gyrA* between positions 3062739 and 3064622 of the *E. coli* O157:H7 *Sakai* genome (accession no. BA000007.2). The PCR reaction was prepared in a 25 μL mixture containing 2.5 μL of 10× PCR buffer (SinaClon, Iran), 2 mM of $MgCl_2$, 50 picomole of each primer, and 50 μM of each ATP, CTP, GTP, and TTP, 2.5 units of *Taq*DNA polymerase (SinaClon, Ekbatan, Tehran, Iran) and 5 μL of extracted DNA. The thermal profile was initiated with a denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 45 s, then the annealing step at 60°C for 45 s, and lastly, the extension step at 72°C for 130 s and again for 5 min. Polymerase chain reaction cycles were performed using a QB-96 gradient thermal cycler (Quanta Biotech, Surrey, England). The PCR products were electrophoresed on 1.5% (w/v) agarose gel containing 2.5 μL per 50 mL of SimplySafe (EURx, Gdańsk, Poland) for 1 h at 75 V and visualized under a UV transilluminator.

Purification of amplified *gyrA* gene and nucleotide sequencing

The amplified *gyrA* gene was purified from the agarose gel using an AmbiClean gel kit (Vivantis, Subang Jaya, Malaysia) according to the manufacturer's instructions. Purified *gyrA* from 6 *E. coli* isolates of different phylogenetic groups were chosen for nucleotide

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Table II. Antimicrobial resistance of *E. coli* from broilers with colibacillosis

	Number (%) of resistant *E. coli* isolates				
Phylogenetic groups	A	B1	B2	D	Total
Antibiotics	(*n* = 16)	(*n* = 17)	(*n* = 23)	(*n* = 28)	(*n* = 84)
Cefazolin	16 (100.0)	11 (64.7)	23 (100.0)	19 (67.8)	84 (100.0)
Imipenem	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Trimetoprim-sulfametoxazol	0 (0.0)	5 (29.4)	22 (95.6)	28 (100.0)	55 (65.5)
Doxycycline	16 (100.0)	16 (94.1)	23 (100.0)	28 (100.0)	83 (98.8)
Colistin	10 (62.5)	0 (0.0)	0 (0.0)	10 (35.7)	20 (23.8)
Soltrim	0 (0.0)	12 (70.5)	23 (100.0)	28 (100.0)	63 (75.0)
Flumequine	5 (31.3)	0 (0.0)	23 (100.0)	28 (100.0)	56 (66.6)
Linco-spectin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Florfenicol	16 (100.0)	12 (70.6)	23 (100.0)	28 (100.0)	79 (94.0)
Enrofloxacin	6 (37.5)	0 (0.0)	0 (0.0)	20 (71.4)	26 (30.9)

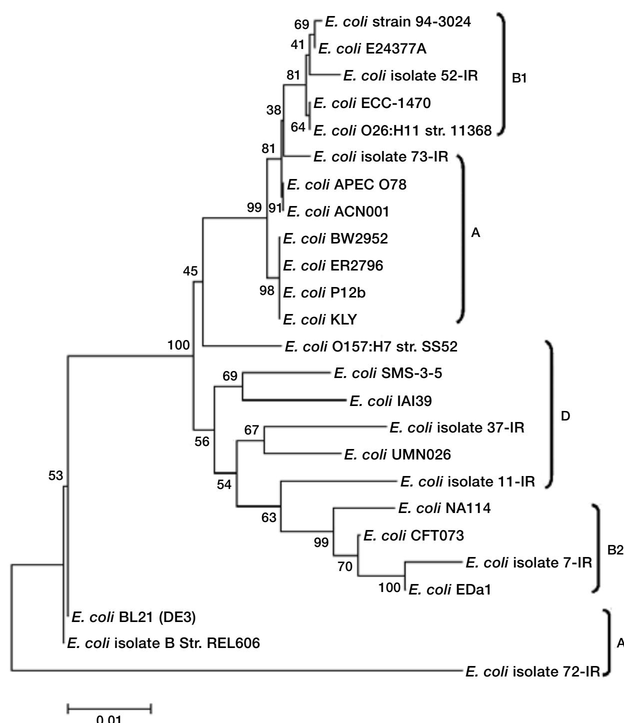

Figure 2. Phylogenetic tree of *gyrA* gene of *E. coli* isolates from chicken with colisepicemia and *E. coli* strains retrieved from GenBank generated using neighbor-joining method in MEGA 6.0.

sequencing and 15 μL of purified PCR products from *E. coli* isolates belonging to each phylogenetic group (A, B1, B2, and D) were sent to SinaClon for sequencing.

gyrA gene nucleotide sequence analysis

Nucleotide sequences were searched in GenBank using the advanced BLAST similarity search option. Nucleotide sequences were aligned and compared with other *gyrA* gene nucleotide sequences from GenBank (Table I) using Clustal W and a phylogenetic tree was generated using the neighbor-joining (NJ) method in MEGA version 6.0 software (15,16).

Table III. Phylogenetic grouping of *E. coli* isolates from chickens with colisepticemia using multiplex PCR and *gyrA* gene nucleotide sequences

	E. coli isolate	Phylogenetic grouping based on multiplex PCR	Phylogenetic grouping based on *gyrA* gene sequences
1	*E. coli* isolate 7-IR	B2	B2
2	*E. coli* isolate 11-IR	D	D
3	*E. coli* isolate 37-IR	D	D
4	*E. coli* isolate 52-IR	B1	B1
5	*E. coli* isolate 72-IR	A	A
6	*E. coli* isolate 73-IR	A	A

Statistical analysis

Statistical analysis was performed with SPSS version 22 (SPSS IBM; Armonk, New York, USA).

Results

Phylogenetic group determination by multiplex PCR

Phylogenetic groups of isolated *E. coli* were determined according to the multiplex PCR method described by Doumith et al (3). Figure 1 shows the amplified *gad*A, *chu*A, *yja*A, and TSPE4.C fragments which were used for phylogenetic grouping. A larger proportion of isolates were classified as group D, comprising 33.33% of all isolates, compared with groups B2, B1, and A, which accounted for 27.38%, 20.24%, and 19.05% of isolates respectively.

Antimicrobial susceptibility

All *E. coli* isolates from the different phylogenetic groups were susceptible to Imipenem and Linco-spectin antibiotics. All *E. coli* isolates showed resistance against Cefazolin, Doxycycline, and Florfenicol, except for 6 isolates from phylogenetic group B1, which

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were sensitive to the antibiotics. All *E. coli* isolates belonging to phylogenetic groups B2 and D were resistant to Soltrim and Flumequine, while most *E. coli* isolates from phylogenetic groups A and B1 were susceptible to them (Table II).

Phylogenetic analysis of isolated *E. coli* based on nucleotide sequences of *gyrA* gene

Nucleotide sequences obtained from 6 *E. coli* isolates from different phylogenetic groups were used to generate a phylogenetic tree. Nineteen *gyrA* gene sequences from different strains of *E. coli* retrieved from GenBank were used for the phylogenetic analysis. Figure 2 shows the phylogenetic tree generated by the MEGA 6.0 program. Based on this phylogenetic tree, *E. coli* isolates were grouped in 5 clusters. Interestingly, *E. coli* strains from phylogenetic groups based on multiplex PCR were clustered within the same phylogenetic groups determined by *gyrA* sequencing, confirming that phylogenetic grouping based on multiplex PCR is in accordance with phylogenetic grouping performed using *gyrA* gene sequence analysis (Table III). *Escherichia coli* strains from phylogenetic group A were divided in 2 different clusters in the constructed tree.

Discussion

Avian colibacillosis has been known for more than a century; however, it still is an endemic disease worldwide (1). In order to understand colibacillosis outbreaks, characterization of *E. coli* population structure based on phylogenetic analysis plays an important role (9). *Escherichia coli* can be classified by a number of distinct phylogenetic groups and it is now obvious that strains in these different phylogenetic groups vary in terms of their ecological niches, life-history characteristics, and propensity to cause disease. Therefore, assigning *E. coli* isolates to one of the recognized phylogenetic groups allows for better understanding of the epidemiology of the diseases caused by this bacteria (4).

Different techniques including multiplex PCR, single strand conformation polymorphism (SSCP) (17), denaturing gradient gel electrophoresis (DGGE) (18), restriction fragment length polymorphism (RFLP) (19), whole genome sequencing, and multilocus sequence typing (MLST) have been employed for the assignment of particular strains to phylogenetic lineages (4,20,21). Sequencing may still be time-consuming and require expertise for interpretation of results, however, it has higher discriminatory power than other rapid genotyping techniques such as SSCP and DGGE which both cover less than 400 bp nucleotide sequences, and RFLP which is based on the variations associated with restriction sites only (22). Multilocus phylogenic lineages will be a more powerful technique for strain identification of *E. coli* isolates (23), however, *gyrA* sequencing may be used as an initial screening tool.

In the present study, *E. coli* isolates from chickens with colibacillosis were assigned to phylogenetic groups based on multiplex PCR and *gyrA* gene sequence analysis. Phylogenetic grouping of *E. coli* isolates based on multiplex PCR and nucleotide sequence analysis of *gyrA* showed that both methods grouped *E. coli* isolates in the same groups.

Escherichia coli isolates belonging to phylogenetic groups A and B1 showed different susceptibility levels to Soltrim and Flumequine compared to *E. coli* isolates from B2 and D phylogenetic groups. This finding may elicit further research on antimicrobial susceptibility differences among different *E. coli* phylogenetic groups. Among the 10 antibiotics used for the antibiogram, *E. coli* isolates showed moderate to high levels of resistance to 8 antibiotics. These results confirm the necessity of antimicrobial susceptibility tests for *E. coli* isolates in order to select appropriate antibiotics for treatment of disease.

The majority of *E. coli* isolates (61.71%) examined in this study belonged to phylogenetic groups B2 and D which supports previous findings that most virulent strains of *E. coli* are in B2 and D phylogenetic groups (24,25). Previous studies in Japan and the United States have shown that phylogenetic groups A and D are predominant among APEC isolates (24,26). In a study by Hiki et al (8), phylogenetic groups A and B1 comprised more that 80% of *E. coli* isolated from healthy broilers, while in the present study these groups accounted for only 39.29% of *E. coli* isolates from APEC. There was a significant difference ($P < 0.01$) between frequencies of phylogenetic groups of *E. coli* isolates from healthy and diseased broilers.

As previously reported, phylogenetic groups A and B1 are recognized as sister groups and in the NJ trees depicted, strains assigned to phylogenetic group A made up a relatively homogeneous group among all the strains assigned to groups A and B1 (4). This finding was confirmed by the NJ tree constructed based on *gyrA* nucleotide sequences in the present study.

In conclusion, *E. coli* isolates from chickens with colibacillosis were distributed in different phylogenetic groups. Moreover, our study suggests that the *gyrA* gene can be used as a candidate marker for phylogenetic grouping of *E. coli.* It also revealed that *E. coli* isolates from chickens with colibacillosis have moderate to high levels of resistance to many antibiotics. Further studies on *gyrA* in *E. coli* in other animals may better clarify the importance of the *gyrA* gene for genotyping *E. coli.*

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Antiviral activity of a novel composition of peracetic acid disinfectant on parvoviruses

Fadi Dagher, Jun Jiang, Peter Tijssen, Jean-François Laliberté

Abstract

Porcine parvoviruses (PPV) are known to be particularly resistant to many disinfectants used to control other non-enveloped viruses. However, effective disinfectants used against PPV are harsh and corrosive to animal health facilities and the environment. We propose a noncorrosive "green" disinfectant that generates peracetic acid *in-situ* and is capable of inactivating PPV completely at a 1% concentration for a 10-minute contact time.

Résumé

Les parvovirus porcins (PVP) sont reconnus pour être particulièrement résistants à plusieurs désinfectants utilisés pour éliminer d'autres virus non-enveloppés. Toutefois, les désinfectants efficaces utilisés contre le PVP sont rudes et corrosifs pour les installations de santé animale et l'environnement. Nous proposons un désinfectant «vert» et non-corrosif qui génère de l'acide peracétique in situ *et qui est capable d'inactiver le PVP complètement lorsqu'utilisé à une concentration de 1 % pour un temps de contact de 10 minutes.*

(Traduit par Docteur Serge Messier)

In the triad of infectious agents, susceptible hosts, and the environment (water, food, contaminated surfaces, aerosols), the role of the latter is the most ambiguous in infectious disease transmission. Viruses may enter the environment in enormous quantities and environmental measures to halt or reduce transmission may offer great prospects for disease control. Ultraviolet (UV) radiation from the sun, if not shielded, is the primary germicide in the environment (1). Virtually all artificial virus inactivation methods are applied locally in laboratories or hospitals in controlled conditions (2,3) since the inhibitors are harsh or cannot be applied over large areas. Notable exceptions are reported and reviewed by Weber and Stlianakis (4) and Vinnerås et al (5). Vinnerås et al (5) reported the effects of formic acid addition to ground high-risk animal by-products (ABP 1) in terms of stabilization and pathogen inactivation. Porcine parvovirus (PPV) was still fully infective after 168 d, even though a strong reaction of formic acid with the single-stranded DNA genome was expected.

Parvoviruses can resist harsh environmental conditions (3), and while surviving on surfaces for long periods they can be transmitted to susceptible hosts. In addition to chemical biocides, heat and irradiation are usually used. Porcine parvoviruses are known to be particularly resistant, since many biocides generally considered as effective against other non-enveloped viruses and used for high-level disinfection have limited inactivation potential. It is a particularly resistant surrogate for inactivation studies of other virus species.

Glutaraldehyde-based disinfectants are used not only in agricultural establishments for animal health but also in hospitals and health care facilities for human safety in order to disinfect surfaces and instruments such as flexible endoscopes (6,7). However, since glutaraldehyde poses an occupational health hazard or risk for staff, with up to 15% of United Kingdom hospitals using it as their first-choice endoscope disinfectant, there is a need for a safer alternative in a bid to reduce potential health, safety, and environmental risks (8,9).

Two percent glutaraldehyde has been the reference disinfectant for high-level disinfection, but its frequent association with adverse effects has stimulated a search for newer disinfectants. When the efficacy of 2% glutaraldehyde was compared with a 0.2% peracetic acid-based disinfectant, both products were effective germicides in 10 to 20 min; however, when organic matter was added, the 0.2% peracetic acid formulation cleaned without corrosion, while 2% glutaraldehyde fixed the matter to the scalpel, causing corrosion within 2 h (10). Also, the PPV inactivation results with 2% glutaraldehyde have been contradictory (3,11). The fixative properties of aldehydes probably decrease due to the presence of organic matter such as soil.

In addition to glutaraldehyde, oxidizers such as chlorine (sodium hypochlorite and chlorine dioxide), hydrogen peroxide, and peracetic acid are also widely used as high-level disinfectants to disinfect surfaces, equipment, and instruments in various industries (12–15). Although they are efficient in controlling pathogens, they are irritants to users (16), corrosive to surfaces, (17) and are not as environmentally friendly as chlorine-based disinfectants (18).

In this study, a patented green inactivator was assessed for its ability to inactivate or mitigate PPV in the environment. First, a sensitive method for the relative infectivity of the virus was developed followed by an assessment of the efficacy.

Porcine testes (PT) fibroblasts, derived from ST cells (ATCC CRL-1746), were grown at 37°C in Dulbecco's modified Eagle's

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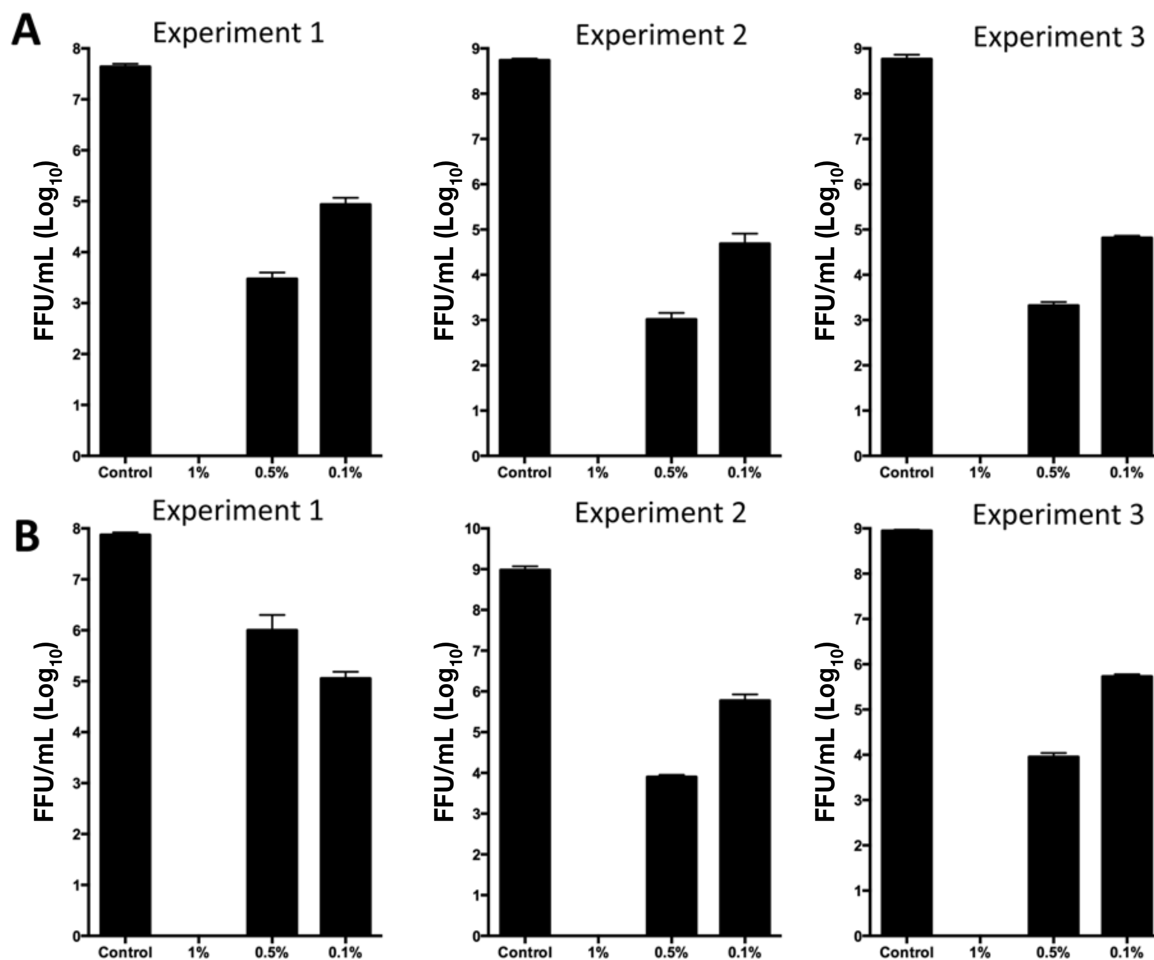

Figure 1. Porcine parvovirus inactivation by BIOXY Enviro or BIOXY +. The PPV virus stocks were incubated with 1%, 0.5%, or 0.1% BIOXY Enviro (A) or BIOXY + (B) for 10 min. The results of repeated experiments are shown.

medium (Wisent, Saint-Bruno, Québec, Canada) containing D-glucose and L-glutamine and supplemented with 7% heat-inactivated bovine serum (Wisent) and antibiotics Penicillin-streptomycin solution (Wisent). The porcine parvovirus (PPV, NADL-2 strain) stock was obtained by propagation in PT cell culture. The viral stocks were collected by a brief centrifugation to remove the cellular debris.

Virus inactivation was done by simply mixing the virus stocks with BIOXY Enviro or BIOXY + solution (Bioxy AFD, Montreal, Quebec, Canada). After incubation with BIOXY Enviro or BIOXY + solution, the virus stocks were mixed vigorously with an equal volume of chloroform-butanol (1:1). The viruses were then recovered by centrifuge at 10 000 rpm for 10 min as described (19). The resulting water phase was collected and loaded into a centrifugal Microcon filter unit (Millipore, Etobicoke, Ontario, Canada) by following the manufacturer's instructions, with the purpose of removing the remaining BIOXY Enviro or BIOXY + compound. The viruses that were retained by the filter were then suspended with equal volume of 1 × phosphate-buffered saline (PBS) for titration.

Viral titers were determined by immunofluorescence (IF) as described previously (20). Briefly, cells were plated at 1×10^4 per well in 96-well plates. The cells were infected 24 h later. The infected cells were proceeded for the IF assay at 20 h after infection. The cells were fixed with 3% formaldehyde solution for at least 30 min, and then were permeabilized with 3% Triton X-100 for 30 min, followed by incubation with a monoclonal mouse capsid-specific antibody (3C9-D11-H11), together with anti-mouse Alexa Fluor 488 as a secondary antibody (Thermo Fischer Scientific, Canada). The fluorescent nuclei were then scored, and the virus titers were expressed in fluorescent focus-forming units/mL (FFU/mL).

First, the PPV virus stocks were treated with 1%, 0.5%, and 0.1% of BIOXY Enviro or BIOXY + for 10 min. The experiments were repeated 3 times. It was found that 1% BIOXY Enviro or BIOXY + inactivated the viruses efficiently, while 0.5% or 0.1% of BIOXY Enviro or BIOXY + showed partial virus inactivation (Figure 1). Additionally, this concentration of BIOXY Enviro or BIOXY + did not affect the viability of the PT cell. No fluorescent signal was detected with cells treated with BIOXY Enviro or BIOXY + (data not shown).

Then, to assess the ability of the BIOXY Enviro and BIOXY + for virus inactivation over a period of 60 min, the PPV virus stock was incubated with 0.1% of BIOXY Enviro or BIOXY + for 10, 20, 30, 40, 50, and 60 min, after which the viral titers were determined. The experiments were repeated 3 times. The viral titers didn't decrease

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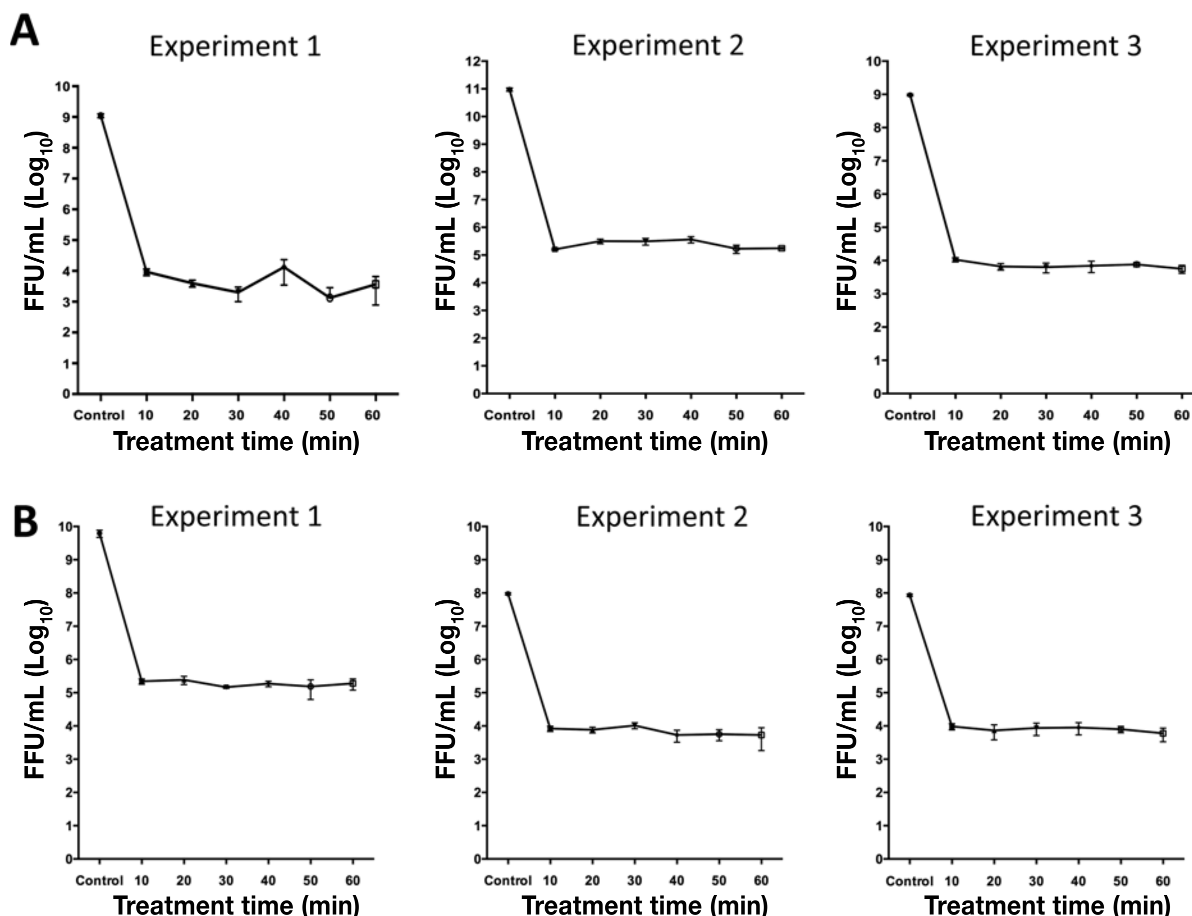

Figure 2. Porcine parvovirus inactivation is concentration-dependent. The PPV virus stocks were treated with 0.1% BIOXY Enviro (A) or 0.1% BIOXY + (B) for 10, 20, 30, 40, 50, and 60 min. The results of repeated experiments are shown.

dramatically after a 10-minute incubation, and the viruses were still infectious even after 60 min (Figure 2). This indicates that the virus inactivation is concentration-dependent.

The PPV were completely inactivated by 1% BIOXY Enviro or BIOXY + for 10 min; however, PPV inactivation was concentration-dependent with the best activity demonstrated at 1% concentration for both BIOXY Enviro or BIOXY +.

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Evaluation of serum myeloperoxidase concentration in dogs with heart failure due to chronic mitral valvular insufficiency

Jong-In Park, Sang-IL Suh, Changbaig Hyun

Abstract

Myeloperoxidase (MPO) is a leukocyte-derived enzyme involved in the process of heart failure and is found to have good diagnostic and prognostic values in humans with chronic heart failure. This study evaluated the relationship between serum MPO levels and the severity of heart failure (HF) due to chronic mitral valvular insufficiency (CMVI) in dogs. Eighty-two client-owned dogs consisting of 69 dogs with different stages of HF due to CMVI and 13 age-matched healthy dogs were enrolled in this study. Serum MPO concentrations in the healthy and CMVI groups were determined by enzyme-linked immunosorbent assay (ELISA) using a canine-specific monoclonal anti-MPO antibody. Serum MPO concentrations were 273.3 ± 179.6 ng/L in the controls, 140.8 ± 114.1 ng/L in the International Small Animal Cardiac Health Council (ISACHC) I group, 109.0 ± 85.2 ng/L in the ISACHC II group, and 106.0 ± 42.3 ng/L in the ISACHC III group. Close negative correlation to serum MPO concentration was found in the severity of heart failure (ISACHC stage). Although this study found a modest relationship between serum MPO levels and the severity of HF due to CMVI in dogs, it also suggested that serum MPO levels decreased as the severity of HF increased.

Résumé

La myéloperoxydase (MPO) est une enzyme dérivée des leucocytes impliquée dans le processus d'une défaillance cardiaque et est reconnue pour avoir une bonne valeur pour le diagnostic et le pronostic chez les humains souffrant d'insuffisance cardiaque chronique. La présente étude a évalué la relation entre les niveaux sériques de MPO et la sévérité de défaillance cardiaque (DC) due à une insuffisance chronique de la valvule mitrale (ICVM) chez des chiens. Quatre-vingt-deux chiens propriété de clients, dont 69 avec différents stades de DC due à une ICVM et 13 chiens en santé jumelés pour l'âge, ont été inclus dans cette étude. Les concentrations sériques de MPO chez les chiens en santé et les groupes de chiens avec ICVM ont été déterminées par épreuve immuno-enzymatique (ELISA) en utilisant un anticorps monoclonal anti-MPO spécifique à l'espèce canine. Les concentrations sériques de MPO étaient de 273,3 ± 179,6 ng/L chez les témoins, de 140,8 ± 114,1 ng/L dans le groupe I du Conseil International de la Santé Cardiaque des Animaux de Compagnie (CISCAS), de 109,0 ± 85,2 ng/L dans le groupe II du CISCAS, et de 106,0 ± 42,3 ng/L dans le groupe III du CISCAS. Une tendance vers une corrélation négative avec la concentration sérique de MPO a été trouvée dans la sévérité de la défaillance cardiaque (stade selon le CISCAS). Bien que dans cette étude une relation modeste entre le niveau sérique de MPO et la sévérité de DC due à une ICVM chez les chiens fut trouvée, il est aussi suggéré que les niveaux sériques de MPO diminuaient à mesure que la sévérité de la DC augmentait.

(Traduit par Docteur Serge Messier)

Introduction

Chronic mitral valvular insufficiency (CMVI) is the most common cause of congestive heart failure (CHF) in small-breed dogs and is characterized by progressive myxomatous degeneration of the atrioventricular valves (1). Several studies have evaluated survival and the prognostic value of biochemical and echocardiographic variables in dogs with CMVI of varying severity (2,3). One recent study reported that some clinical signs (e.g., dyspnea and syncope) and echocardiographic indices (e.g., end-diastolic volume-index, left atrial to aortic root ratio, and E-wave transmitral peak velocity) were significantly associated with survival time in dogs with CMVI (1).

Myeloperoxidase (MPO) is a leukocyte-derived enzyme involved in lipid peroxidation, the direct scavenging of nitric oxide, and nitric oxide synthase inhibition (4). Recent studies found that plasma MPO levels have good diagnostic and prognostic value in humans with chronic heart failure (5,6). Myeloperoxidase (MPO) was shown to have a good predictive value for adverse cardiac events, e.g., non-fatal myocardial infarction, death, and the need for revascularization, in patients presenting with either chest pain (7) or acute coronary syndromes (8). Studies on mice also found that an elevation in plasma MPO levels was closely linked to inflammation, oxidative stress, and impaired cardiac remodelling (9,10).

The primary objective of this study was to determine the relationship between serum MPO levels and the severity of HF due to CMVI in dogs. We hypothesized that elevated levels of MPO might be observed across varying stages of HF and be closely related to other prognostic indicators, e.g., left atrium (LA) to proximal aortic (Ao) diameter ratio (LA:Ao), left ventricular dimension at systole (LVID):Ao (LVID:Ao) diameter ratio, and transmitral E-peak velocity.

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Materials and methods

Study population

Prior to this study, we obtained the approval of the animal ethics committee of Kangwon National University to collect blood samples from healthy dogs for serum MPO assays. Informed written consent for sample collection, including information pertinent to our investigation, was obtained from the dog owners before the study began. Our study population consisted of 2 groups. The normal control group consisted of 13 healthy, small-breed dogs (2.8 to 9.0 kg) from 7 to 14 y of age that had no evidence of cardiovascular or other systemic diseases, as revealed by physical examination, complete blood (cell) count (CBC), chemistry panel, chest radiography, and echocardiography. The CMVI age-matched group included 69 small-breed dogs (2.4 to 11.2 kg) from 7 to 17 y of age. All dogs with CMVI had undergone a cardiology consultation due to previous identification of a heart murmur or the presence of clinical signs indicating a cardiovascular disorder, including cough, exercise intolerance, or both. All dogs underwent physical examination, echocardiography, CBC, and a chemistry panel. We excluded dogs that had other clinically relevant systemic diseases, such as renal failure and hypertension. Chronic mitral valvular insufficiency (CMVI) was diagnosed based on clinical signs, chest radiography data, and echocardiographic findings, according to the guidelines for diagnosis of CMVI in dogs (11). The dogs with CMVI were divided according to criteria proposed by the International Small Animal Cardiac Health Council (ISACHC) for the functional classification of heart failure. Some dogs with symptomatic CMVI were receiving medications, such as enalapril, furosemide, spironolactone, pimobendan, digoxin, and amlodipine, for heart disease depending on its severity.

Analysis of serum myeloperoxidase concentration

To minimize the influence of a meal, all dogs were fasted for 12 h before collecting their blood samples. Whole blood was withdrawn from either the cephalic or jugular vein to determine serum levels of MPO. Blood samples were drawn directly into sterile Vacutainer tubes (BD Diagnostics, Franklin Lakes, New Jersey, USA) and then centrifuged at $1.500 \times g$ for 10 min at 4°C. The supernatants were stored at −80°C or on dry ice for shipping. The serum MPO levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA)-based kit (Dog MPO ELISA Kit; BioSource, San Diego, California, USA), according to the manufacturer's recommendations.

Briefly, standards and samples were pipetted into the wells and the MPO present in the samples was bound to the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for MPO was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was added to the wells. After washing to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells; color developed in proportion to the amount of MPO that bound during the initial step. The color development was stopped and the intensity of the color was measured. Before the study, the test kit and method were completely validated for use with dog serum and serial dilutions of the samples were tested in duplicate.

Echocardiography

All echocardiographic examinations were carried out by a board-certified cardiologist (Hyun) in accordance with the recommended standards for dogs. Motion mode (M-mode), Doppler, and 2-dimensional (2-D) echocardiography were conducted in left and right lateral recumbency. Motion mode echocardiography was used to measure the left ventricular dimension at systole (LVIDs) and diastole (LVIDd). Two-dimensional echocardiography was used to measure the diameter of the left atrium (LA) and the proximal aortic (Ao) from the right parasternal short axis at the aortic valve level. Pulsed-wave Doppler echocardiography was used to measure the transmitral E-peak velocity from the left parasternal apical 4-chamber plane at the tips of the mitral valve when open. These measurements were used to determine the LA to proximal Ao diameter (LA:Ao) ratio and the LVIDd to Ao diameter (LVIDd:Ao) ratio.

Statistical analysis

Statistical analyses were carried out using commercially available statistical software (SPSS Version 21; SPSS, Chicago, Illinois, USA). Descriptive statistics were calculated for quantitative variables by study group and analyzed for normality using the Kolmogorov-Smirnov test. Differences in serum MPO concentrations among groups were evaluated using the Kruskal-Wallis one-way analysis of variance (ANOVA). The Pearson's coefficient for bivariate correlation analysis was used to test the strength of the association between i) serum MPO concentration and other echocardiographic indices used to evaluate the severity of HF, and ii) serum MPO concentration and age, body weight, and gender. In all comparisons, a probability value of $P < 0.05$ was considered statistically significant, unless stated otherwise.

Results

The mean serum MPO concentration in this study population was 273.3 ± 179.6 ng/L in the control group ($n = 11$), 140.8 ± 114.1 ng/L in the ISACHC I group ($n = 19$), 109.0 ± 85.2 ng/L in the ISACHC II group ($n = 29$), and 106.0 ± 42.3 ng/L in the ISACHC III group ($n = 20$). The mean serum MPO concentration was much lower in CMVI groups. Furthermore, the mean serum MPO concentration in dogs with advanced heart failure (ISACHC II and III groups) was approximately 2.5 times lower than that in healthy control dogs. A statistically significant difference was found between the control and the CMVI group ($P < 0.05$) and between the ISACHC I and ISACHC II and III groups ($P < 0.05$) (Figure 1). Using a univariate analysis, serum MPO levels were found to be correlated with ISACHC stage ($r = -0.607$, $P = 0.005$) and LVID:Ao ratio ($r = -0.200$, $P = 0.04$). However, there was no significant correlation between serum MPO level and age ($r = 0.224$, $P = 0.121$), body weight ($r = 0.151$, $P = 0.299$), LA:Ao ratio ($r = 0.157$, $P = 0.191$), E-peak ($r = 0.07$, $P = 0.552$), or LA diameter ($r = -0.225$, $P = 0.06$) (Figure 2).

Discussion

Recent human studies found that plasma MPO levels were closely correlated to the severity of HF and mortality in patients with acute

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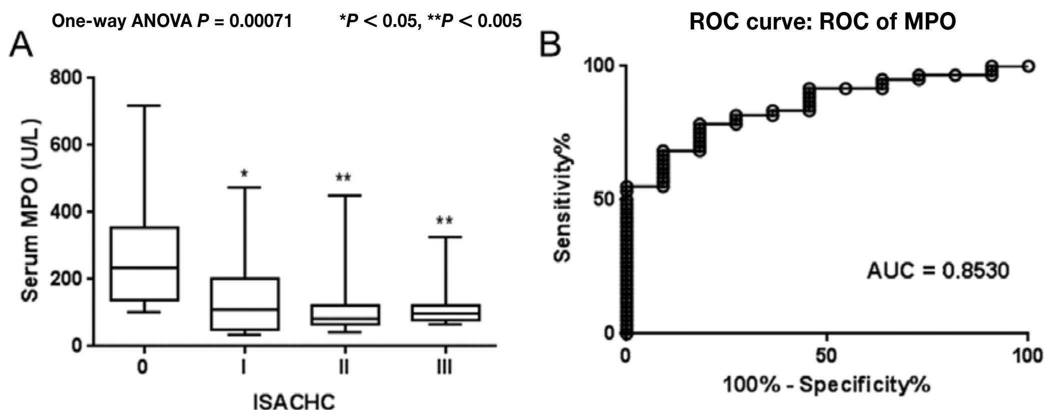

Figure 1. Serum myeloperoxidase (MPO) levels in this study population. A — The median concentration was significantly lower in dogs with chronic mitral valvular insufficiency. Statistically significant differences were found between the control and the ISACHC I, II, and III groups; statistically significant differences were found between the ISACHC I and the ISACHC II and III groups. $^*P < 0.01$; $^{**}P < 0.05$. B — The sensitivity and specificity of serum MPO for detecting heart failure in dogs with advanced stages of heart failure were high.

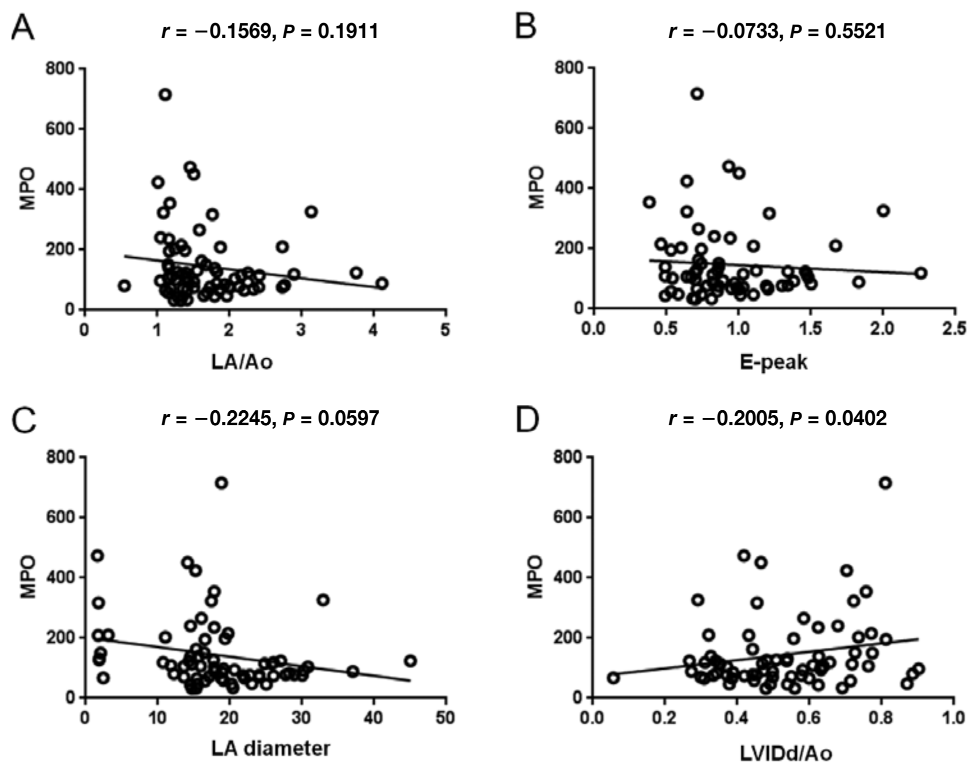

Figure 2. The correlation between serum myeloperoxidase (MPO) level and echocardiographic variables.

coronary disease and chronic systolic HF (5,6). Mouse studies with a coronary artery ligation model and an ischemia reperfusion model also found that MPO might be involved in pathological processes such as inflammation, oxidative stress, and cardiac remodelling (9,10). In humans, the MPO test is widely used to screen for cardiac diseases (7). Unlike human studies, this study found a negative correlation between serum MPO level and the severity of HF in dogs with CMVI. The serum MPO level decreased as the HF worsened in our study population. The serum MPO level was not closely correlated with echocardiographic indices, however, which indicates worsening of HF, i.e., LA:Ao ratio and E-peak, although the LVIDd:Ao ratio was weakly correlated with the serum MPO. As serum MPO reference ranges have yet to be determined in healthy dogs, further study is required to verify whether the MPO concentration was actually normal in the control group and reduced in the CMVI group.

The discrepancy in the diagnostic value of the MPO between human and canine studies might be due to differences in the pathophysiology of HF in dogs. Unfortunately, no veterinary studies have evaluated the role of MPO in the pathogenesis of HF progression.

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Furthermore, MPO testing or accepted cutoff values for serum MPO levels in dogs have not yet been standardized. Therefore, the serum MPO level in dogs with HF might be different from that in human studies. In addition, the MPO test is a nonspecific test and the MPO level can be increased in several other types of diseases, i.e., inflammatory, infectious, and infiltrative diseases (12). Because our healthy control group was an aged population, the dogs in this group might have had occult diseases that were not evident in routine laboratory examination. Recent human studies also found that periodontal disease could cause an increase in serum MPO concentration (13), although this study did not evaluate the dental hygiene status of the enrolled population. Because our control group was age-matched, we expected the control and CMVI groups to have similar levels of dental hygiene status so that it would only minimally affect our study result.

Because most dogs with CMVI were on cardiac medications, e.g., diuretics, pimobendan, or angiotensin converting enzyme, the lowered serum MPO levels in dogs with advanced HF might be due to the effects of these drugs or to normalization of myocardial damage and remodelling with treatment. However, this hypothesis that dogs with advanced heart failure had lower MPO concentrations because of medication may not be correct. One recent study found that leveosimendan, a drug similar to pimobendan, had anti-inflammatory effects in mice (14).

Our last hypothesis for the discrepancy between studies in humans and in dogs is that the incidence of coronary diseases is generally much lower in dogs than in humans. Since several human studies have suggested that MPO plays a key role in the process of atherosclerosis, which later leads to acute and chronic HF (6,8), the lower incidence of coronary diseases in dogs might cause this discrepancy. The diagnostic value of MPO testing in humans is limited to heart diseases associated with oxidative stress and inflammation (15), while the mitral valvular degeneration in our study model had a different pathological mechanism than these human heart diseases. This different mechanism in canine valvular disease might be another reason why the serum MPO was lowered with the progression of HF in this study population.

Our study had several limitations. First, the study population was not large enough to obtain the statistical power needed to find an accurate diagnostic value for MPO in dogs with HF due to CMVI. Secondly, the level of serum MPO in this study might be influenced by diet and medication, which were different in the control and CMVI groups. Further studies are necessary to determine the effects of diet and medication, e.g., pimobendan, on serum MPO levels in dogs. Lastly, correlation studies with other biomarkers, e.g., cardiac troponin and N-terminal probrain natriuretic peptide, were not attempted in this study. Further study with these markers would be beneficial in understanding the role of MPO in canine heart failure due to CMVI.

In conclusion, this study evaluated the relationship between serum MPO levels and the severity of HF due to CMVI in dogs. We found that serum MPO levels decreased as the severity of HF increased. A different pathophysiology in canine heart disease and the effects of diet and medication might be the cause of discrepancies between canine and human studies. Further studies are required to validate the findings of this study.

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The effects of oral administration of Yunnan Baiyao on blood coagulation in beagle dogs as measured by kaolin-activated thromboelastography and buccal mucosal bleeding times

Jami Frederick, Søren Boysen, Catherine Wagg, Serge Chalhoub

Abstract

We examined the effects of oral administration of Yunnan Baiyao (YB) on hemostasis by measuring buccal mucosal bleeding times (BMBTs) and doing citrated kaolin-activated whole-blood thromboelastography (TEG). In a randomized controlled crossover trial 8 beagle dogs were given either placebo or 1000 mg of YB orally every 12 h for 5 consecutive treatments. Blood was drawn 24 h before treatment and 2 and 24 h after the last treatment, and the BMBT was measured in each sample in duplicate. The TEG analysis was done in duplicate 60 ± 5 min after sample collection. There were no adverse effects of treatment and no significant differences between the control and treatment BMBTs or TEG parameters at any time point. Significant differences were found between baseline and 24 h after the last treatment within the treatment group for the TEG parameters LY30 and LY60 and within the control group for the TEG parameters MA, G, LY30, and LY60. Thus, at the dose and frequency of administration in this study YB did not appear to have any clinically significant effects on the measured coagulation parameters. The differences within the treatment group were likely due to analytic error since similar differences were seen in the control group. Further studies with a larger sample, as well as more direct measures of platelet function, are needed.

Résumé

Nous avons examiné les effets de l'administration orale de Yunnan Baiyao (YB) sur l'hémostase en mesurant le temps de saignement de la muqueuse buccale (TSMB) et en faisant une thromboélastographie (TEG) de sang entier après activation par de la kaoline citratée. Lors d'un essai en croisé randomisé et contrôlé, huit chiens beagle ont reçu soit un placebo ou 1000 mg de YB par voie orale à chaque 12 h pour cinq traitements consécutifs. Du sang a été prélevé 24 h avant le traitement et 2 et 24 h après le dernier traitement, et le TSMB mesuré dans chaque échantillon en duplicata. L'analyse TEG a été faite en duplicata 60 ± 5 min après le prélèvement de l'échantillon. Il n'y eut aucun effet néfaste du traitement et aucune différence significative entre le groupe témoin et le groupe traité pour ce qui est des TSMBs ou des paramètres de la TEG à tous les points d'échantillonnage. Des différences significatives ont été trouvées entre les valeurs de base et 24 h après le dernier traitement à l'intérieur du groupe traité pour les paramètres LY30 et LY60 de la TEG et à l'intérieur du groupe témoin pour les paramètres MA, G, LY30 et LY60 de la TEG. Ainsi, à la dose et à la fréquence d'administration utilisées dans la présente étude, YB ne semble pas avoir d'effet clinique significatif sur les paramètres de coagulation mesurés. Les différences dans le groupe traité sont fort probablement dues à une erreur analytique car des différences similaires ont été notées dans le groupe témoin. Des études supplémentaires avec un échantillonnage plus grand, ainsi que des mesures plus directes de la fonction des plaquettes sont requises.

(Traduit par Docteur Serge Messier)

Introduction

Yunnan Baiyao (YB) is a Chinese herbal supplement that has been used for its supposed hemostatic properties for almost a century. Knowledge of its specific properties is limited, but that has not deterred many physicians and veterinarians from employing it in various hemorrhagic and pathological conditions (1–4). The use of YB in canine veterinary practice is already widespread despite there being no reports of studies examining the efficacy of YB in reducing the incidence of hemorrhage in dogs.

The first studies that attempted to determine the efficacy of YB used laboratory animals as models. Topical administration of YB was shown to decrease bleeding times in rats and blood clotting times in rabbits (5,6). Another study showed that orally administered YB could shorten *in-vivo* bleeding time in rats when a piece of liver was excised and *in-vitro* blood clotting time in rabbits (7). It was also shown that YB could induce ultrastructural changes in platelets and platelet-constituent release, which possibly accounted for its hemostatic effects (8). Some later studies using humans as subjects determined that YB significantly reduced perioperative bleeding as well as bleeding in various ulcerative–hemorrhagic conditions (1,3,4).

Prior studies have found *in-vivo* bleeding times, such as those produced with liver laceration or tail transection, and template bleeding time (TBT) to be the most useful measures of YB efficacy (1,5–7,9). The results of other common coagulation tests, such as prothrombin time (PT), activated partial thromboplastin time (aPTT), and the d-dimer test, were not affected in human patients or in dogs treated with YB (1,10). The activated clotting time (ACT) was also not affected in ponies treated with YB (9). Citrated kaolin-activated whole-blood thromboelastography (TEG) has only once before been

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used to measure the efficacy of YB, with no significant results, but may be useful because of its ability to assess all coagulation parameters, including fibrinolysis (10).

The objective of this study was to determine the effects of oral administration of YB on coagulation in 8 beagle dogs as measured by TEG and buccal mucosal bleeding times (BMBTs). Our hypothesis was that this treatment would result in TEG tracings with decreased R and K values and decreased lytic parameters, as well as a shortened BMBT as compared with control treatment. The R value represents the reaction time; that is, the time until the first evidence of a clot is detected. The K value is the time from the end of R until the clot reaches 20 mm in diameter; this represents the speed of clot formation.

Materials and methods

Eight university-owned beagle dogs (5 male and 3 female) were used in this study. Inclusion criteria included normal results of a physical examination, a normal initial complete blood (cell) count (CBC) and biochemistry panel, and a normal coagulation profile as measured by BMBT and TEG analysis. The dogs were between 5 and 8 y old, and their mean body weight (BW) was 10.7 ± 1.5 kg (extremes 8.9 and 12.8 kg). Two of the dogs received cyclosporine ophthalmic ointment daily throughout the study for treatment of chronic keratoconjunctivitis sicca. Animal care protocols were approved by the University of Calgary Veterinary Sciences Animal Care Committee (VSACC). Protocol number AC13-0266

The dogs were individually housed in runs overnight and then spent about 8 h in group outdoor yards. The 3 youngest dogs spent outdoor time together, as did the 5 oldest. Seven of the dogs were fed a standard commercial dog food twice per day, whereas the dog that was overweight was fed a low-calorie commercial dog food twice per day.

The dogs were randomly assigned to 2 groups: YB and control. The researchers were blind to the group assignments until after data analysis. The YB group was treated with 4 × 250 mg (1000 mg) of YB divided equally into 2 Greenies Pill Pockets [Nutro Products (a subsidiary of Mars Incorporated), Franklin, Tennessee, USA]. The control group received 2 empty Pill Pockets. Treatment was every 12 h for 5 doses. For ease of dosing and blood sampling the dogs were managed in pairs, 2 h apart.

Blood was collected 24 h before the first treatment (baseline) and 2 h and 24 h after the last treatment. With the use of 20-gauge 1-inch Monoject needles and 6-mL Monoject syringes (Medtronic, Minneapolis, Minnesota, USA) 2 mL of blood was collected from alternating jugular veins; that is, if the first attempt to obtain a blood sample failed, then the contralateral jugular vein was used. The blood was immediately transferred into sterile BD Vacutainer tubes containing 3.2% sodium citrate (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The tubes were filled to the maximum fill line to ensure a 9:1 ratio of blood to anticoagulant, were inverted 5 times to allow mixing of blood and anticoagulant, and were kept at room temperature until TEG analysis was started, 60 ± 5 min after collection.

The BMBT was measured 24 h before the first treatment (baseline) and 2 and 24 h after the last treatment. The lancets used to create a small, standardized wound in the buccal mucosa were single-use disposable devices (Surgicutt; International Technidyne Corporation, Piscataway, New Jersey, USA). Three people were present for each test, one to restrain the dog in lateral recumbency, one to hold the lip up as well as to start and stop a timer, and one to use the lancet. In one case the dog's excessive head movement necessitated securing the lip with a piece of roll gauze. The buccal mucosa was dried with a towel if excessive saliva was present. The timer was started when the lancet was used. Blotting paper was used to blot blood as it accumulated at the periphery of the incision while avoiding the incision itself to prevent disruption of the clot. The timer was stopped when the blotting paper no longer absorbed blood from the periphery. The incision was repeated on the other side of the mouth, and the average of the 2 times was recorded for each dog.

A 10-day washout period was allowed, after which a crossover study was done in the same manner.

The TEG analysis was done at 37°C in duplicate simultaneously on the same Thromboelastograph Hemostasis Analyzer (Haemonetics, Braintree, Massachusetts, USA). The average of the 2 values was used for statistical analysis. In brief, 1 mL of citrated whole blood was transferred to room-temperature vials containing 40 μL of kaolin. The tubes were inverted gently 5 times to allow mixing of blood and activator; 340 μL of this mixture was pipetted into TEG cups containing 20 μL of room-temperature 0.2 M calcium chloride, and the TEG analysis was started. The TEG analyses were run until the R and K values, the angle between R and K (α-angle), the maximum amplitude (MA), the shear elastic modulus strength (G), and the percentage clot lysis at 30 and 60 min (LY30 and LY60) were finalized. Electronic tests along with level I (normal tracing) and level II (hypocoagulable tracing) control tests were run every morning of analysis to ensure accurate results.

Statistical analysis

All results were tested for normalcy with the d'Agostino and Pearson Omnibus Normality Test. For all data sets that passed this test, within-group comparisons were done by 1-way repeated-measures analysis of variance (ANOVA) with a Dunnett's multiple-comparisons test. For all data sets that failed to pass the normalcy test, a repeated-measures Friedman's test was used with a *post-hoc* Dunn's multiple-comparisons test. For between-groups comparisons a 2-way ANOVA and *post-hoc* Bonferoni test were used.

Results

Both the initial and crossover phases of the trial were completed with all 8 dogs. No adverse effects were noted throughout the study according to the results of physical examination. All TEG controls had results within the reference ranges throughout the study. All data passed the normalcy test with the exception of R and α-angle.

There were no significant between-group differences in the means of any of the parameters measured in this study. The 95% confidence intervals (CIs) of the mean differences (Table I) demonstrated a large range of values, and all included the zero-difference value.

In the control group there was a significant within-group difference between the mean values for baseline and 24-hour MA ($P = 0.04$) and G ($P = 0.03$); the *post-hoc* Dunnett's test showed means of 56.08 ± 4.517 (standard deviation) and 6.513 ± 1.113, respectively,

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Table I. Mean differences in buccal mucosal bleeding time (BMBT) and thromboelastography parameters in 8 dogs before and after treatment with Yunnan Baiyao and before and after a control period during a randomized controlled crossover study

Time or	Difference	Time in relation to treatment		
parameter	between groups	24 h before	2 h after	24 h after
BMBT (s)	Mean	8.88	8.38	−10.5
	95% CI	−24.85 to 42.6	−25.35 to 42.1	−44.22 to 23.22
	P-value	> 0.99	> 0.99	> 0.99
R (min)	Mean	0.32	−0.58	−0.13
	95% CI	−0.7 to 1.33	−1.6 to 0.43	−1.14 to 0.9
	P-value	> 0.99	0.48	> 0.99
α-angle (°)	Mean	2.18	1.78	−1.54
	95% CI	−3.46 to 7.8	−3.85 to 7.41	−7.18 to 4.09
	P-value	> 0.99	> 0.99	> 0.99
K (min)	Mean	−0.21	−0.21	0.09
	95% CI	−0.76 to 0.35	−0.76 to 0.35	−0.47 to 0.64
	P-value	> 0.99	> 0.99	> 0.99
MA (mm)	Mean	0.7	−1.73	−2.93
	95% CI	−4.48 to 5.88	−6.91 to 3.45	−8.11 to 2.25
	P-value	> 0.99	> 0.99	0.5
G (dynes/s)	Mean	0.21	−0.37	−0.9
	95% CI	−1.09 to 1.5	−1.66 to 0.92	−2.19 to 0.39
	P-value	> 0.99	> 0.99	0.27
LY30 (%)	Mean	0.15	0.57	0.9
	95% CI	−5.21 to 5.51	−4.79 to 5.93	−4.46 to 6.26
	P-value	> 0.99	> 0.99	> 0.99
LY60 (%)	Mean	1.21	1.67	0.44
	95% CI	−6.87 to 9.28	−6.4 to 9.74	−7.63 to 8.51
	P-value	> 0.99	> 0.99	> 0.99

CI — confidence interval; R — reaction time; α-angle — angle between R and K; K — time from 2 to 20 mm in amplitude; MA — maximum amplitude; G — shear elastic modulus strength; LY30 — percent clot lysis at 30 min; LY60 — percent clot lysis at 60 min.

at baseline and 52.93 ± 2.473 and 5.663 ± 0.5662, respectively, at 24 h ($P = 0.05$ for both). The control group also showed a significant within-group difference between the mean values for baseline and 24-hour LY30 ($P < 0.01$) and LY60 ($P = 0.02$); the *post-hoc* Dunnett's test showed means of 2.369 ± 3.625 and 6.719 ± 7.334, respectively, at baseline and 7.438 ± 3.744 and 12.79 ± 4.714, respectively, at 24 h ($P < 0.05$ for both).

In the YB group there was a significant within-group difference between the mean values for baseline and 24-hour LY30 ($P = 0.03$) and LY60 ($P = 0.02$, respectively); the *post-hoc* Dunnett's test showed means of 2.219 ± 3.148 and 5.513 ± 5.507 at baseline and 6.538 ± 5.422 and 12.35 ± 6.058 at 24 h ($P < 0.05$ for both).

There were no significant within-group differences in BMBT, R, K, or α-angle.

Discussion

This study aimed to obtain more information on how YB affects coagulation in canines through the use of *in-vivo* and *in-vitro* methods. We ultimately chose to use the BMBT to evaluate *in-vivo* bleeding times because many earlier studies used similar *in-vivo* bleeding times, such as liver and tail transection and TBT, and they reported significant differences between treatment and control groups (5–7,9). However, in our study there was no significant difference between treatment and control groups in BMBT or in the TEG values. The 95% CI of the mean between-group differences revealed a large range of values that included zero difference for all the parameters. These collective results suggest that YB, at the dose and frequency of administration that we used, did not have a measurable effect on coagulation in the 8 dogs studied.

There were significant within-group differences between baseline and 24 h for the LY30 and LY60 TEG values in our study; however, the fact that these changes occurred in both the control and treatment groups argues against the differences being due to the administration of YB. There were no significant within-group changes in BMBT.

The results of this study do not correlate with those in the current, limited YB literature, in which reported studies in both animals and humans have yielded results suggesting that YB has a measurable

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effect on coagulation, particularly on bleeding times (1,4–7,9). It is uncertain why in this study the dogs did not demonstrate any observable differences, but this could be due to observer error, patient-related factors, product variation, dosage inadequacy, species differences, or small sample size.

The BMBT has been shown to be a useful *in-vivo* test of primary hemostasis, but multiple studies have shown that there is great interobserver and intraobserver variation in BMBTs recorded on the same animal (11,12). To try to minimize variation in this study, the same observer conducted all BMBT tests on all animals. However, some intraobserver variability could have been partially responsible for the statistical insignificance of the results. This could have been due to the relative inexperience of the observer, although the experimenter had practised on 2 dogs to refine the technique. The observer was blinded to treatment assignments until after data analysis to eliminate any bias when recording times.

Animal-related factors could have played a role in the variability of measurements as well. There were times when dogs struggled against the restraint, which could have led to increased blood pressure and subsequent disruption of the forming clot. However, there were always 3 people assisting to minimize patient-behavior factors.

Variability can be seen when different-sized BMBT devices are used (13). In the present study all BMBT devices were the same size throughout the study to minimize obvious variation of incision size. We chose the manufacturer-recommended device size for use in patients of this weight category.

Another reason for the lack of difference between groups may be related to the lack of quality control in the production of YB. This nutraceutical is unregulated and therefore could vary from batch to batch. Owing to logistical difficulties we were forced to use YB bought at different times from different sources. It could have been manufactured at very different times, and the ingredients and amounts could have varied in a manner that rendered some of the batches less efficacious.

The package insert for the YB used recommends oral administration of 1 to 2 of the 250-mg capsules up to 4 times per day in humans. There are no specific dosage recommendations published for animals. The dosage used in this study (1000 mg per 5- to 15-kg dog twice daily) was based on the results of a previous clinical study in dogs (10) and our clinical experience that most practitioners use 2 to 3 times the manufacturer-recommended dose. A study in rats yielded significant results when the dose was 240 mg/kg, much higher than that used in this study (7). It is possible, therefore, that the dosage we chose was too low for an effect to be seen. Without prior studies evaluating the efficacy of oral YB treatment on hemostasis in dogs, the administration and sampling times in this study were based on trials in humans and other animals that observed clinical effects of oral YB treatment. In rabbits and rats a significant hemostatic effect was observed according to the BMBT starting 30 min after administration of YB and persisting till at least 4 h after administration (7). The duration of efficacy of orally administered YB in dogs has not yet been established, and extrapolation from other species may have led to sampling times in our study that did not reflect peak hemostatic effects.

Another important consideration is that it is not known if there are species variations in the efficacy of YB. It is possible that canines do not respond in the same way as humans and small rodents. Further studies are needed in domestic animals to determine whether there are species differences in the response to YB.

An important limitation of the present study is the relatively small sample size. With only 8 dogs, the power in this study was limited and the range of values representing the difference of the means between groups quite large. Clinical trials with larger samples might be able to detect smaller differences between groups and provide a narrower range of values wherein the true mean difference between groups would lie. However, the clinical importance of these ranges of mean differences in TEG parameters remains difficult to fully interpret.

Another consideration is the fact that 2 of the dogs used in this study were receiving topical cyclosporine treatment of chronic keratoconjunctivitis sicca. Systemic administration of cyclosporine can induce a hypercoagulable state in humans (14). Oral administration of cyclosporine to dogs has been shown to alter the platelet membrane and is associated with a significant increase in thromboxane production (15). There are no published reports of studies that showed that topical administration yielded the same effect in canines. The results of the present study do not seem to indicate that the 2 dogs that were given cyclosporine had any significant changes in their coagulation parameters as compared with the other dogs. The TEG tracings for these 2 dogs indicated normal coagulation, and the BMBTs were comparable to those of the other dogs, which suggests that the topical application of cyclosporine was not responsible for a hypercoagulable state in these dogs.

The significant differences in the TEG values in both the control and treatment groups do not appear to be due to the administration of YB, as within-group significant differences in LY30 and LY60 were present in both groups. There could be multiple explanations as to why these differences occurred. The TEG machines are extremely sensitive to preanalytic and analytic factors (16). Even though measures were taken to decrease the presence of these factors, such as using a standardized sample-collection technique, having a single person collecting and analyzing all the samples, and using standardized temperatures of analyses, there still could have been some variation in preanalytic and analytic techniques that led to these differences. The TEG analysis for all samples was done at 37°C, as the reference intervals for kaolin-activated canine samples were determined at this temperature (17). Because the physiological temperature of the healthy canine is normally between 38°C and 39°C, there may have been some differences in clotting kinetics with the lower temperature of analysis. This temperature was chosen for ease of comparison with reference intervals.

A recent study in dogs found that TEG was not a sensitive indicator of hypercoagulable states and suggested that a shortened PT/PTT maybe a more sensitive indicator in some situations (17). It is possible that the dogs used in our study in fact had hypercoagulable states but the tests used were unable to detect this. However, a study investigating oral YB administration in dogs at a lower dose than used in the current study failed to show a significant difference in PT, aPTT, fibrinogen, and d-dimer values when compared with the values in control dogs (10).

Reference intervals have been established for kaolin-activated samples in canines (18). All of the mean values in the present study fell within these intervals with the exception of those for LY30 and

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LY60, for which reference intervals were not available for canine patients. Although the within-group differences for MA and G were significant, they fell within these established reference ranges. Thus, the differences in MA and G in the control group were likely of no clinical significance and could have been due to preanalytic and/or analytic variation as discussed.

In the present study, financial and time constraints limited platelet function analysis to the use of BMBT. Further studies could include more direct measures of platelet function. It has been determined that YB can cause the release of platelet constituents and other ultrastructural changes (8). It would have been interesting to see under the electron microscope how canine platelets respond to YB. Other tests that might have helped to determine the nature of YB's effect on platelets could have included TEG platelet mapping, as this technique has been shown to detect hypercoagulable states in canines (19). Another useful test would have been platelet aggregometry, as this has been found to be useful in testing platelet function in dogs (20).

At the dosage and frequency of administration used in this study, YB seems to be safe for dogs. Physical examination yielded no change in results from baseline. It is unknown, however, if YB has any effect on blood parameters that are routinely measured, as these were assessed only in the initial phase, before dosing. More in-depth studies are needed to confirm the safety of the product in terms of the complete blood count and biochemical parameters after dosing.

In conclusion, Yunnan Baiyao, at the dosage used in this study (1000 mg per 5- to 15-kg dog twice a day), given orally for 5 consecutive treatments, appears to be safe for canines. It did not produce a significant reduction in BMBT or changes in TEG results, including the R and K values and fibrinolysis, in a comparison of the control and YB groups.

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Article

Association of gingivitis with dental calculus thickness or dental calculus coverage and subgingival bacteria in feline leukemia virus- and feline immunodeficiency virus-negative cats

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Abstract

Periodontal disease is the most common oral disease in cats. The objectives of this study were to determine the relationships between gingivitis and dental calculus thickness (DCT), or dental calculus coverage (DCC); determine the association of gingivitis scores and types of oral bacteria; and to evaluate bacterial co-infection in cats with periodontal disease. Twelve cats that were not infected with feline leukemia or feline immunodeficiency viruses were enrolled in the study. Gingivitis, DCT, and DCC were scored and recorded. A Kruskal-Wallis test was used to compare scores among canine, 2nd premolar, 3rd premolar, 4th premolar, and 1st molar teeth. The relationship between gingivitis and DCT or DCC scores was determined using the Spearman rank sum test (ρ). Subgingival bacteria were cultured and the association between bacterial species and gingivitis score was evaluated using a Fisher's exact test. The average gingivitis, DCT, and DCC scores for the caudal maxillary teeth were higher for the caudal mandibular teeth and more severe for the 3rd premolar, 4th premolar, and 1st molar teeth than for the canine teeth. A strong relationship between average DCT or DCC score and average gingivitis score was found (ρ = 0.96 and 1, respectively). Aerobic and anaerobic bacterial infections were identified in a large number of cats with periodontal disease (71.1% and 28.9%, respectively). In conclusion, severe gingivitis scores were associated with anaerobic bacterial infection. The caudal teeth are affected with more severe gingivitis, DCT, and DCC than the other teeth. Antibiotic prophylaxis should be prescribed in cats with periodontal disease.

Résumé

La maladie parodontale est la maladie orale la plus fréquente chez les chats. Les objectifs de la présente étude étaient de déterminer les relations entre la gingivite et l'épaisseur du tartre dentaire (ETD), ou la couverture du tartre dentaire (CTD); déterminer l'association des pointages de gingivite et les types de bactéries orales; et d'évaluer les co-infections bactériennes chez les chats avec maladie parodontale. Douze chats qui n'étaient pas infectés par le virus de la leucémie féline ou le virus de l'immunodéficience féline ont été recrutés pour cette étude. La gingivite, l'ETD, et la CTD ont été évalués et notés. Un test de Kruskal-Wallis a permis de comparer les pointages parmi les dents canines, 2e prémolaires, 3e prémolaires, 4e prémolaires et 1ère molaires. La relation entre la gingivite et les pointages d'ETD ou de CTD a été déterminée à l'aide du test de corrélation de Spearman (ρ). Les bactéries sous-gingivales ont été cultivées et l'association entre les espèces bactériennes et le pointage de gingivite a été évaluée à l'aide du test exact de Fisher. Les pointages moyens de gingivite, d'ETD, et de CTD pour les dents maxillaires caudales étaient plus élevés que pour les dents mandibulaires caudales et plus sévères pour les dents 3e prémolaires, 4e prémolaires, et 1ère molaires que pour les canines. Une forte relation entre les pointages moyens d'ETD et de CTD et les pointages moyens de gingivite a été trouvée (ρ = 0,96 et 1, respectivement). Des infections bactériennes aérobies et anaérobies ont été identifiées chez un grand nombre de chats avec maladie parodontale (71,1 % et 28,9 %, respectivement). En conclusion, des pointages élevés de gingivite étaient associés avec des infections par des bactéries anaérobiques. Les dents caudales étaient plus sévèrement affectées de gingivite, d'ETD, et de CTD que les autres dents. Une antibiothérapie prophylactique devrait être prescrite pour des chats avec maladie parodontale.

(Traduit par Docteur Serge Messier)

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Introduction

Periodontal disease, an inflammation of the tissues and supportive structures surrounding the teeth, is one of the most common oral diseases in cats (1–4). The periodontium consists of gingiva, periodontal ligament, alveolar bone and cementum (1–4). Continued inflammation can lead to gingival recession and damage to the cementum, periodontal ligament, and/or alveolar bone (5,6), resulting in loss of teeth. The clinical signs of periodontal disease in cats include halitosis, drooling, pain, discomfort, facial swelling, nasal discharge, gingivitis, accumulation of dental calculus, mobile teeth, inappetence, and loss of teeth (2,3,5). The periodontal disease may have a systemic impact that could result in impairment of other organs such as chronic kidney disease, cardiovascular disease, and septicemia (6–10).

Accumulation of bacteria and plaque on the surface of teeth is suggested to be an underlying culprit for periodontal disease (3). Plaque is a white or yellowish biofilm that is formed by bacteria on the teeth (1), whereas dental calculus is formed by the mineralization of minerals in saliva on soft plaque. The inflammation of gingiva may erupt from irritation caused by bacterial toxins from plaque, inflammation from dental calculus deposition, or the inflammation of other periodontal tissues. Dental calculus and gingivitis cause the destruction of teeth and surrounding tissues, which can lead to severe periodontal disease and loss of teeth (6). Certain oral bacteria, including *Fusobacterium* spp., *Eubacterium* spp., and *Peptostreptococcus* spp., have been found in cats with periodontal diseases (11,12). Thus, feline periodontitis may be linked to the presence of specific bacterial species.

To the best of our knowledge, no study has focused on the relationship between gingivitis, dental calculus thickness (DCT), dental calculus coverage (DCC), and oral bacterial infection in cats without feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV). Since FeLV and FIV may affect the host immune response and compromise the normal gingival tissue function (13), only cats which were free of FeLV and FIV infection were selected for the present study. This study sought to i) evaluate the severity of gingivitis, DCT, and DCC for different teeth; ii) determine the putative correlations between gingivitis score and DCT or DCC score in cats without FeLV and FIV; iii) determine the correlation between gingivitis score and type of oral bacteria; and iv) evaluate cultivable bacterial co-infection and drug sensitivity in cats with periodontal disease.

Materials and methods

Twelve cats, 8 female and 4 male, were randomly selected and enrolled in this study during professional dental cleaning procedures. The median age of cats was 5 y (min-max range: 1 to 10 y). Six cats were domestic shorthaired cats and the other 6 were Persians. All cats were negative for FeLV antigen and FIV antibody as assessed by a commercial enzyme-linked immunosorbent assay (ELISA) test (SNAP FIV/FeLV Combo Test; IDEXX Laboratories, Maine, USA). The experimental procedure for this study was approved by the Kasetsart University Animal Committee (ID number: ACKU04859).

Table I. Scoring system for gingivitis, dental calculus thickness, and dental calculus coverage scores

Score	Description
Gingivitis score	
Score	Description
0	normal
1	no gingival bleeding on probing examination
2	gingival bleeding on probing examination
3	severe inflammation; gingival hypertrophy and spontaneous gingival bleeding
Dental calculus thickness score	
Score	Description
0	absence of dental calculus
1	dental calculus thickness < 0.5 mm
2	dental calculus thickness 0.5 to 1.0 mm
3	dental calculus thickness > 1.0 mm
Dental calculus coverage score	
Score	Description
0	absence dental calculus
1	dental calculus coverage at supragingival margin
2	moderate coverage of dental calculus at supragingival and subgingival margin
3	wide coverage of dental calculus at supragingival and subgingival margin

All cats underwent general anesthesia with diazepam (Diapine; Atlantic Pharmaceutical, Bangkok, Thailand), 0.02 mg/kg body weight (BW), IV as a premedication and 1% propofol (Anepol injection; Hana Pharmaceutical, Seoul, South Korea), 2 to 4 mg/kg BW, IV for anesthetic induction. The general anesthesia was maintained using 2% isoflurane (Attane; Piramal Critical Care, Pennsylvania, USA) in oxygen. An oral examination was performed on each cat and gingivitis, DCT, and DCC were scored using a modified Silness and Löe scoring system (Table I). All scores were evaluated by a single veterinary dentist (14). The scores were noted for each tooth and then were combined by tooth type and recorded, as canine, 2nd premolar, 3rd premolar, 4th premolar, and 1st molar teeth. The scores for 3rd premolar, 4th premolar, and 1st molar teeth of both the upper and the lower jaw were combined as caudal maxillary teeth and caudal mandibular teeth, respectively.

The gingivitis score ranged from 0 to 3, based on the degree of inflammation and bleeding. The gingival score was determined using a sterile blunt probe (Probe CP10; Dental USA, Illinois, USA). A score of 0 indicated a normal gingiva and no inflammation. A score of 1 indicated the presence of mild inflammation of the gingiva, a slight change in color and edema, and no bleeding upon probing. A score of 2 indicated moderate inflammation of the gingiva with the presence of gingival bleeding upon probing. Finally, a score of 3 indicated severe inflammation of the gingiva, severe gingival redness and swelling with the presence of severe and spontaneous gingival bleeding. Gingivitis scores of 0 and 1 were combined as less clinically relevant gingivitis and gingivitis scores of 2 and 3 were combined to a more clinically relevant gingivitis group to determine a possible association among bacterial groups and the severity of gingivitis scores.

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Table II. The median (min to max range) for gingivitis scores, dental calculus thickness (DCT) scores, and dental calculus coverage (DCC) scores for each tooth

Dental scores	Canine	2nd Premolar	3rd Premolar	4th Premolar	1st Molar
Gingivitis					
Upper teeth	1.0 (0.5 to 2.0)	1.0 (0 to 2.0)	1.8 (1.0 to 3.0)	2.5 (1.0 to 3.0)	2.8 (2.0 to 3.0)
Lower teeth	1.0 (0 to 1.5)	N/A	1.0 (0 to 3.0)	1.8 (0 to 3.0)	2.0 (0 to 3.0)
Dental calculus thickness					
Upper teeth	1.0 (0 to 2.0)	1.0 (0 to 2.0)	2.5 (0 to 3.0)	3.0 (0 to 3.0)	3.0 (2.0 to 3.0)
Lower teeth	0.8 (0 to 1.0)	N/A	1.0 (0 to 3.0)	1.3 (0 to 3.0)	1.5 (0 to 3.0)
Dental calculus coverage					
Upper teeth	1.0 (0 to 2.0)	1.0 (0 to 3.0)	1.8 (0.6 to 3.0)	2.5 (0.5 to 3.0)	1.5 (0 to 3.0)
Lower teeth	1.0 (0 to 3.0)	N/A	1.0 (0 to 3.0)	1.8 (0 to 3.0)	2.0 (0 to 3.0)

N/A — Not available — the mandibular 2nd premolar tooth is absent in cats.

Dental calculus thickness scores ranged from 0 to 3. A score of 0 reflected the absence of dental calculus. A score of 1 indicated a dental calculus thickness of up to 0.5 mm, while a score of 2 indicated a dental calculus thickness ranging from 0.5 to 1.0 mm. Finally, a score of 3 indicated a dental calculus thickness of more than 1 mm.

Dental calculus coverage scores were characterized on a scale of 0 to 3. A score of 0 indicated the absence of dental calculus. A score of 1 indicated mild calculus coverage at the supragingival margin (up to 1 mm). A score of 2 indicated a moderate amount of supragingival and subgingival calculus, while a score of 3 indicated wide calculus coverage in both the supragingival and subgingival areas.

Aerobic and anaerobic bacteria were sampled using a sterile absorbent endodontic paper points (Paper points; Shanghai Dental, Bangkok, Thailand). The paper points were inserted and left in the subgingival area for 30 s near the left maxillary and mandibular 4th premolar teeth. The paper points were immediately stored in Amies Transport Medium (Difco Transport Medium Amies; Becton Dickinson, Franklin Lakes, New Jersey, USA) and thioglycolate broth (Fluid thioglycolate medium; Himedia Laboratories Pvt., Mumbai, India) as transport media for both aerobic and anaerobic bacteria, respectively. Aerobic bacteria were cultured on blood agar (BBL blood agar base (infusion agar); Becton Dickinson, Franklin Lakes, New Jersey, USA) and MacConkey agar (Difco MacConkey agar; Becton Dickinson), while the anaerobic bacteria were cultured on thioglycolate medium and anaerobe basal agar (Anaerobe basal agar; Oxoid, Hampshire, England). Drug sensitivities of the bacteria were determined using an elution test.

A commercially available software package (GraphPad Prism version 5.0; GraphPad Software, La Jolla, California, USA) was applied to the data for statistical analysis. A normality test for each score was performed using the Shapiro-Wilk W-test. The severities of gingivitis, DCT, and DCC scores for each tooth were compared using a Kruskal-Wallis test. Dunn's post-test was used to determine differences among groups. The 3 scores of the caudal maxillary teeth and the mandibular teeth were also compared using a Mann-Whitney U-test. The Spearman rank sum correlation test (ρ) was applied to determine the relationship between the gingivitis score and DCC or DCT score. Fisher's exact test also was applied to evaluate the association between bacterial groups and the severity of gingivitis score. Results were considered to be statistically significant at $P < 0.05$.

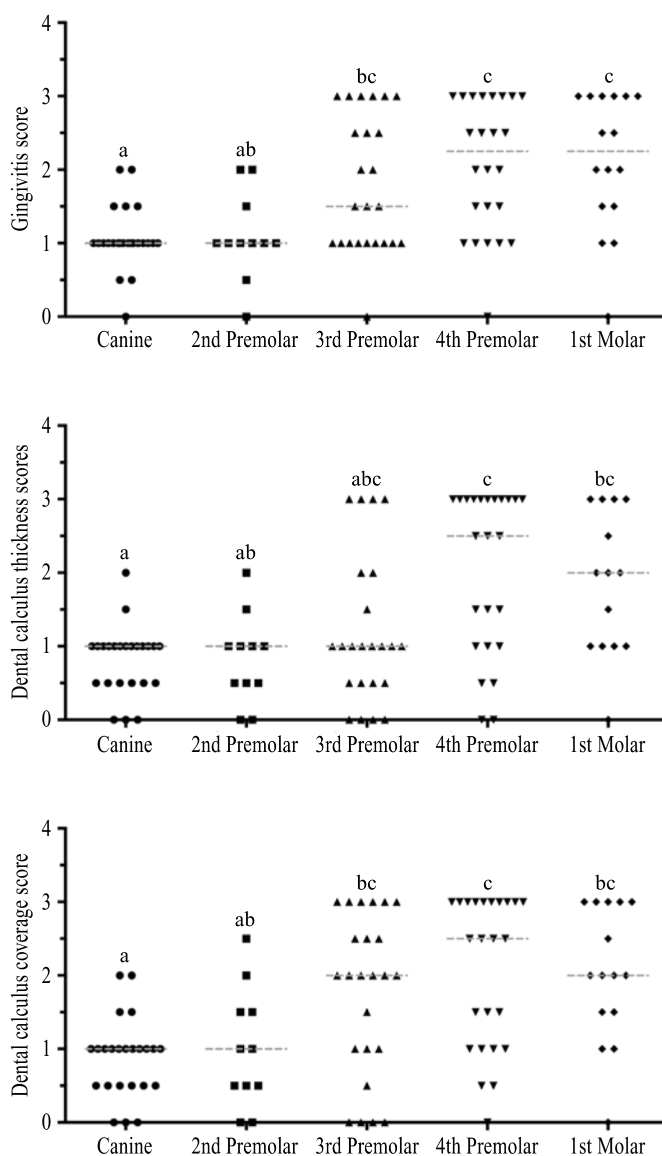

Figure 1. Gingivitis, dental calculus thickness (DCT), and dental calculus coverage (DCC) scores for each tooth evaluated. The medians of each score are shown in dashed lines. Columns not sharing a common script are significantly different ($P < 0.05$).

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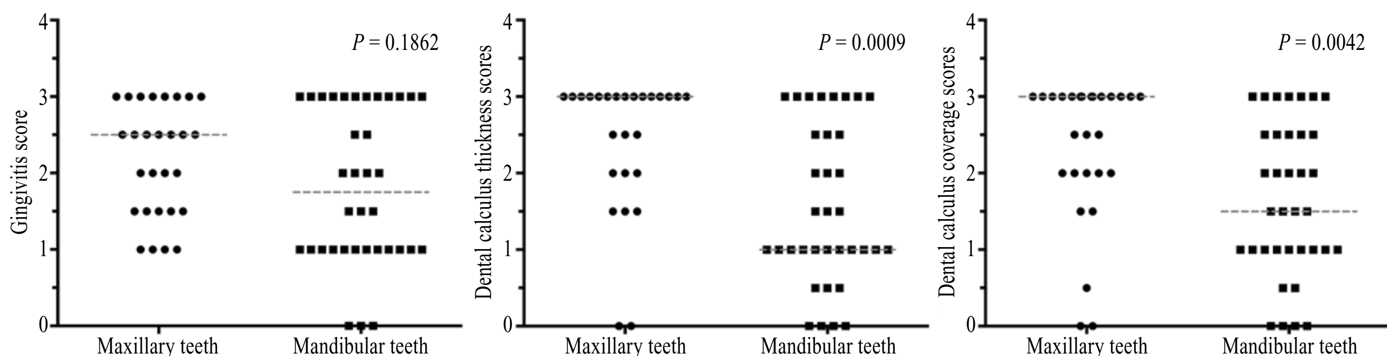

Figure 2. Comparison of gingivitis, dental calculus thickness (DCT), and dental calculus coverage (DCC) scores of caudal maxillary and mandibular teeth.

Results

The median and range of gingivitis, DCT, and DCC scores for all teeth are shown in Table II. The difference in gingivitis, DCT, and DCC scores for different teeth were statistically significantly different ($P < 0.0001$, 0.0002, and 0.0001, respectively; Figure 1). Dunn's post-test analysis revealed that gingivitis scores were significantly lower for canine teeth than for 3rd premolar, 4th premolar, or 1st molar teeth ($P = 0.0388$, 0.0007, and 0.0013, respectively). The gingivitis scores for 2nd premolar teeth were significantly lower than those for the 4th premolar or 1st molar teeth ($P = 0.0264$ and 0.0256, respectively). Dental calculus thickness scores were significantly higher for 1st molar teeth than for canine teeth ($P = 0.0243$). Dental calculus thickness scores were significantly higher for 4th premolar teeth than for the canine or 2nd premolar teeth ($P = 0.0014$ and 0.0222, respectively). Finally, DCC scores were significantly lower for canine teeth than for the 3rd premolar, 4th premolar, or 1st molar teeth ($P = 0.0255$, 0.0007, and 0.0050, respectively). Dental calculus coverage scores were significantly lower for 2nd premolar teeth than for 4th premolar teeth ($P = 0.0456$).

The gingivitis scores for caudal maxillary teeth were not significantly different from the gingivitis scores for caudal mandibular teeth ($P = 0.1862$), whereas the DCT and DCC scores for caudal maxillary teeth were more severe than those for caudal mandibular teeth ($P = 0.0009$ and 0.0042, respectively; Figure 2).

A strong relationship between gingivitis scores and DCT scores was found ($\rho = 0.8862$; $P < 0.0001$). In addition, a strong relationship between gingivitis scores and DCC scores was found ($\rho = 0.8758$; $P < 0.0001$), as shown in Figure 3.

The bacterial culture from the gingival sulcus of the left maxillary and mandibular 4th premolar teeth from 12 cats revealed 71.1% and 28.9% aerobic and anaerobic bacterial species, respectively, as shown in Table II. The aerobic bacteria identified in this study were *Pasteurella multocida, Streptococcus* spp., *Enterococcus* spp., *Staphylococcus* spp., *Bacillus cereus, Escherichia coli,* and *Pseudomonas aeroginosa.* Meanwhile, anaerobic bacterial species identified included *Bacteroides* spp., *Peptostreptococcus anaerobius,* and *Eubacterium aerofaciens. Bacteroides* spp. was a commonly identified anaerobic bacterium in cats with higher gingivitis scores ($P = 0.0278$). Associations between bacterial groups and gingivitis scores are shown in Table III.

Antimicrobial sensitivity testing for all anaerobic bacteria species identified in this study is shown in Table IV. All of the anaerobic bacteria were sensitive to clindamycin, chloramphenicol, metronidazole, cefoxitin, or tetracycline. *Peptostreptococcus anaerobius* and *Eubacterium aerofaciens* were sensitive to erythromycin and penicillin. *Pasteurella multocida,* the most abundant aerobic bacterial species, was sensitive to cefoxitin in all cats.

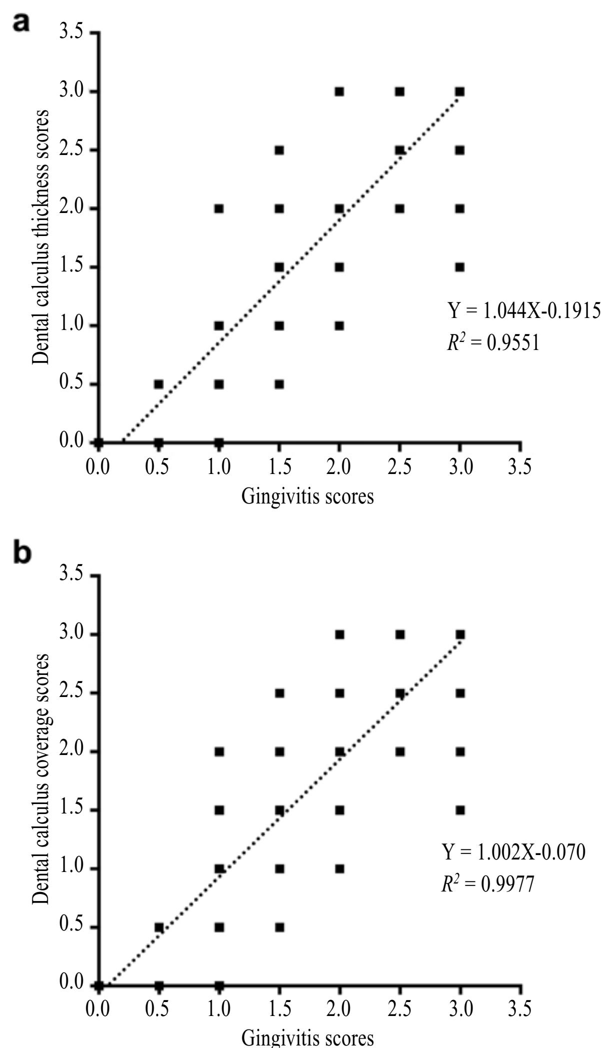

Figure 3. The linear relationships between gingivitis scores and dental calculus thickness (DCT) or dental calculus coverage (DCC) scores.

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Table III. Aerobic and anaerobic bacterial culture results from samples collected from the gingival sulcus of the left maxillary and mandibular 4th premolar in 12 cats and the association between bacterial groups and gingivitis scores

Bacterial groups	Bacterial species	Number of cats (% of total bacterial species identified)	Gingivitis scores 0 to 1 (*n* = 7)	Gingivitis scores 2 to 3 (*n* = 5)	Fisher's exact test (*P*-value)
Aerobic bacteria	*Pasteurella multocida*	10 (26.3%)	7	3	0.1515
	Streptococcus spp.	7 (18.5%)	3	4	0.2929
	Enterococcus spp.	4 (10.5%)	1	3	0.2222
	Staphylococcus spp.	3 (7.9%)	2	1	0.5500
	Bacillus cereus	1 (2.6%)	0	1	0.5500
	Escherichia coli	1 (2.6%)	0	1	0.5500
	Pseudomonas aeruginosa	1 (2.6%)	0	1	0.5500
Anaerobic bacteria	*Bacteroides* spp.	7 (18.5%)	2	5	0.0278
	Peptostreptococcus anaerobius	3 (7.9%)	0	3	0.1058
	Eubacterium aerofaciens	1 (2.6%)	0	1	0.5500

Table IV. Antimicrobial sensitivity for anaerobic bacteria that were identified from the left maxillary and mandibular 4th premolar gingival sulcus in cats

Antimicrobials	*Bacteroides tectum* (*n* = 3)	*Bacteroides buccae* (*n* = 2)	*Bacteroides oralis* (*n* = 1)	*Eubacterium aerofaciens* (*n* = 1)	*Peptostreptococcus anaerobius* (*n* = 3)	Number of cats (%)
Penicillin	S	R	S	S	S	8 (80%)
Cefoxitin	S	S	S	S	S	10 (100%)
Clindamycin	S	S	S	S	S	10 (100%)
Erythromycin	S	S	R	S	S	9 (90%)
Chloramphenicol	S	S	S	S	S	10 (100%)
Tetracycline	S	S	S	S	S	10 (100%)
Metronidazole	S	S	S	S	S	10 (100%)

S — susceptible; R — resistant.

Discussion

Periodontal disease has been reported as the most common disease to cause health issues in cats with a prevalence of 13.8% of the cat population (4). In the present study, the association of gingivitis with dental calculus and bacterial infection was evaluated in 12 cats undergoing routine dental cleaning. Accumulation of dental calculus was identified in premolar and 1st molar teeth with the presence of gingivitis. Bacterial culture as well as antimicrobial sensitivity testing was also performed on samples from the gingival sulcus. Interestingly, high gingivitis scores in our cats were significantly associated with the identification of anaerobic bacterial species.

The incisors were not evaluated herein because they are small in cats and commonly lost. These teeth would not be representative of common dental problems found in a clinic. The rostral and caudal teeth were divided by the occlusion of the upper and lower teeth. The interdigitate occlusion was identified on the canine teeth (maxilla and mandible) and 2nd premolar (maxilla) teeth. In addition, the maxillary dental arch is slightly wider than the mandibular dental arch, so that the maxillary 3rd premolar, 4th premolar, and 1st molar teeth slightly overlap those of the mandibular teeth. The results of this study indicate that the canine and 2nd premolar teeth have less severe gingivitis, and lower DCT, and DCC scores than the 3rd premolar, 4th premolar, and 1st molar teeth. This could be explained by the openings of the ducts from the parotid and zygomatic salivary glands that are located near the 4th premolar and the 1st molar teeth (15,16). Mineral deposition from the saliva in this area may cause dental calculus formation. Furthermore, dental calculus formation may lead to the irritation of gingiva thus causing gingivitis.

Dental calculus thickness and DCC scores were significantly higher for the caudal maxillary teeth than for the caudal mandibular teeth. This difference can be attributed to the anatomical occlusion of the caudal maxillary teeth and the caudal mandibular teeth. Because the maxillary dental arch is wider than the mandibular dental arch in cats, the mechanical scrubbing action during mastication helps lower dental calculus build-up of the caudal mandibular teeth.

The relationship between gingivitis scores and DCT or DCC scores was positively correlated. This may support the idea that dental calculus in cats is followed by the development of gingivitis (6). However, the severity of gingivitis may not solely depend on DCT and DCC. In this study, the gingivitis scores for the caudal upper teeth did not differ significantly from those for the caudal lower teeth, suggesting that the underlying causes of gingivitis are multifactorial. As reported in previous studies, cats with resorptive lesions, dental calculus, food allergies, and infectious diseases, such as FeLV, FIV, feline calicivirus, and oral bacteria are predisposed to developing gingivitis (12,17–19).

The aim of this study was to determine the association of gingivitis with DCT or DCC and subgingival bacterial infection. The periodontal pocket depth and oral radiographic evaluation were not

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fully performed to determine a periodontitis condition; however, the significance of this study should not be affected because bacterial-induced gingivitis has been reported in non-plaque, plaque, or periodontitis conditions (20,21).

The development of gingivitis in cats infected with FeLV and FIV was not evaluated in the present study. Cats infected with either FeLV or FIV are more susceptible to periodontal disease because of the impaired immune system that may further compromise the gingiva of cats that already have dental calculus and bacterial infections (12,22). To eliminate gingivitis that may have been attributed in part to FeLV or FIV infection, all cats with these infections were excluded from this study. One limitation of the present study is that the cats were not tested for feline calicivirus infection. However, calicivirus infections also cause severe stomatitis (23), which was not identified in any of the cats in this study.

Thioglycolate broth is primarily developed for determining the oxygen requirement of microorganisms and also to aid in the isolation of obligate anaerobes when used as a transport medium (24,25). Since sodium thioglycolate in the transport medium utilizes oxygen in the medium, it protects the obligate anaerobes from oxygen damage. Various studies indicated the crucial role of anaerobic infection as an underlying culprit of gingivitis and periodontitis, thus it is recommended that thioglycolate broth be applied as a transport medium, especially in feline patients with severe gingivitis.

The results of this study resemble those of other studies. It has been reported previously that *Bacteroides* spp. and *Peptostreptococcus anaerobius* were isolated from cats with severe gingivitis scores and that *Pasteurella multocida* was isolated from most samples with lower gingivitis scores (26). Similarly, this study found *Bacteroides* spp. in cats with high gingivitis scores. This study also identified *Pasteurella multocida* in 10 of the 12 cats (7 cats with lower gingivitis scores and 3 cats with higher gingivitis scores); however, there was no statistical association between *Pasteurella multocida* and gingivitis scores. Consistent with the present study, anaerobic bacterial infection has been shown to be a risk factor for severe gingivitis (3). Specialized transport media served as an important tool for helping to identify and increase the chance of isolating anaerobic bacteria in our study.

Antibiotic treatment is recommended as an adjunctive treatment for cats after professional dental cleaning and in cats with periodontitis (27,28). The drug sensitivity results in this study suggest that cats with severe gingivitis scores may be given cefoxitin, clindamycin, chloramphenicol, tetracycline, or metronidazole as a first-line antibiotic agent for the treatment of anaerobic bacteria. Routine dental care also should be encouraged to slow down plaque formation and the development of future periodontitis.

In conclusion, the findings of this study indicate that the caudal maxillary and mandibular teeth of cats tend to have more severe gingivitis, DCT, and DCC scores than other teeth. Therefore, cat owners and veterinarians should pay more attention to these areas during dental care. Anaerobic bacterial infection also plays a crucial part in the development of gingivitis in cats. Antibiotic prophylaxis for feline dental procedure is also recommended to minimize risk of local as well as systemic infectious complications originated from gingival bacteria. Future research should be aimed at investigating factors contributing to gingivitis in cats.

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Computed tomographic assessment of a new nonsurgical sinus trephination technique using a medical bone marrow drill

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Abstract

The objective of this study was to determine the feasibility of trephination of the frontal sinus and injection of antifungal cream using a medical bone marrow drill in dogs. Results were compared with frontal sinus trephination using a standard surgical technique. Bilateral trephination of the frontal sinuses was carried out in the heads of 11 cadavers using a medical bone marrow drill and a surgical bone chuck. The time taken to carry out the procedure using both techniques was compared. Before and after injection of antifungal cream into the frontal sinuses, computed-tomography (CT) scanning was done to assess for iatrogenic trauma and to determine the degree to which the sinuses were filled with each technique and evaluate the diffusion of the cream into the nasal cavity of each dog. The mean volume of the sinuses was 8.8 mL (3.1 to 14.3 mL). Trephination, flushing, and injecting of antifungal cream were significantly faster using the medical technique. There was no significant difference in the mean filling of the frontal sinuses between the medical (82.7%) and the surgical (82.4%) technique (*P*-value = 0.3). Filling of the nasal cavity was classified as very good in 6/11 cases, with evidence of trauma caused by the surgical trephination technique in 1 head. Findings suggest that use of the medical bone marrow drill is highly feasible for frontal sinus trephination. Injection of antifungal cream into the frontal sinuses using the bone marrow needle resulted in good diffusion into the ipsilateral nasal cavity and could be used to treat aspergillosis when debridement or sinusoscopy is not deemed necessary.

Résumé

*L'objectif de cette étude est d'évaluer l'utilisation d'une perceuse de moelle osseuse pour la trépanation du sinus frontal et l'injection de crème antifungique chez le chien. Les résultats sont comparés à la trépanation du sinus par une technique chirurgicale classique. Les sinus frontaux de 11 têtes de cadavres de chiens ont été trépanés, en utilisant une perceuse pour moelle osseuse ou un trépan chirurgical. La durée de la procédure avec chaque technique est comparée. Les sinus frontaux sont examinés par tomodensitométrie avant et après injection de crème antifungique, afin de noter de potentielles lésions iatrogéniques, d'évaluer le degré de remplissage du sinus frontal pour chaque technique, et d'apprécier la diffusion de la crème dans les cavité nasales pour chaque chien. Le volume moyen des sinus était de 8,8 ml (3,1 à 14,3 ml). La trépanation, le flush et l'injection de crème antifungique étaient significativement plus rapide avec la perceuse médicale. Il n'y avait pas de différence significative entre le remplissage moyen des sinus par la technique médicale (82,7 %) ou chirurgicale (82,4 %) (*P *= 0,3). Le remplissage des cavités nasales était qualifié de très bon dans 6 cas sur 11. Une lésion iatrogénique fut constatée dans un cas, avec la technique chirurgicale. Ces résultats semblent montrer qu'une perceuse médicale pour moelle osseuse est facilement utilisable pour trépaner le sinus frontal chez le chien. L'injection de crème dans le sinus frontal par le trocart de la perceuse pour moelle osseuse permet un bon remplissage de la cavité nasale ipsilatérale, et pourrait donc être utilisée dans le cadre du traitement de l'aspergillose canine, dans les cas où un débridement du sinus n'est pas nécessaire.*

(Traduit par Docteur Serge Messier)

Introduction

Canine sinonasal aspergillosis is a relatively common opportunistic infection of the upper respiratory tract with a worldwide distribution. It is most commonly caused by an *Aspergillus* species and affects primarily the nasal cavity. The frontal sinuses can be secondarily involved when the infection moves through 1 or both nasofrontal ostia. While the anatomy of the canine frontal sinuses varies greatly among individual dogs and breeds, they are typically divided into rostral, medial, and lateral compartments that are usually each connected separately to the ipsilateral nasal cavity (1).

The optimal therapy for sinonasal aspergillosis has yet to be determined despite having evolved a lot through the years. Systemic administration of an antifungal medication was originally the standard treatment, but this is costly, requires prolonged administration, presents risk of serious adverse systemic side effects, e.g., hepatotoxicosis, anorexia, and vomiting, and has a poor success rate for cure, ranging from 40% to 50% with ketoconazole and

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thiabendazole to 60% to 70% with fluconazole and itraconazole (2–5). A higher success rate for cure has been reported with topical treatment, although prolonged contact between the medication and the infected nasal mucosa is required to achieve clinical efficacy. The first topical treatment protocol consisted of surgically placed indwelling catheters into the frontal sinuses, followed by continuous infusion of enilconazole solution for 7 to 14 d (6). Clinical cure was reported in up to 90% of the patients using this protocol (4). This technique has been abandoned in favor of less invasive techniques due to long hospitalization periods, discomfort, and premature removal of the catheters by treated dogs.

Another protocol was later described in which enilconazole solution was infused for 1 h using a catheter placed through the nostril. This was carried out on anesthetized dogs placed in lateral recumbency and results showed up to 65% of patients reached a clinical cure after 1 treatment and 87% after 1 or more treatments (7,8). This technique was further refined with 10% enilconazole solution being deposited by endoscopically placing catheters directly into the sinuses, with cure rates of 85% to 100% reported after a single treatment and 100% after 2 to 3 treatments (9,10).

As infusion of the solution is very time-consuming and often has to be repeated, research was conducted into using a depot of an antifungal cream. Because of its viscosity, the cream persisted longer in the nasal cavity than a solution (11). A study showed an 86% cure rate in treating nasal aspergillosis with a 5-min infusion of the nasal cavity with enilconazole solution followed by instillation of clotrimazole cream into the frontal sinuses by temporary trephination, even when the disease was limited to the nasal cavity (12). Infusion of bifonazole cream into the frontal sinuses through endoscopically placed catheters after preliminary infusion of enilconazole solution for 1 h showed good results (cure rate of 70% and 100% after 1 and 2 treatments, respectively) and using a depot of the bifonazole cream alone resulted in a cure rate of 70% after a single treatment (13). This procedure requires good technical skills in order to catheterize the frontal sinuses endoscopically.

While temporary trephination has been proven effective for instilling an antifungal solution providing there is extensive contact between the mucosal surfaces of the frontal sinuses and nasal cavities, retention of the solution appears to be poor (14). A more recent study on cadavers has shown excellent distribution of the clotrimazole cream in the frontal sinuses and caudal nasal cavity when administered by surgical trephination of the sinuses (15).

In future, treatment of aspergillosis will probably rely on instilling an antifungal cream into the frontal sinuses in order to achieve prolonged exposure of the mucosa to the antimycotic product, assuming that subsequent diffusion of the cream in the sinuses and nasal cavity is adequate. This technique can even be used when the sinuses are not involved, in which case the slow elimination of the cream through the nasal cavity allows the nasal turbinates to be exposed to the antimycotic medication for a prolonged period (12).

The objective of this study was to assess the feasibility and duration of trephination of the frontal sinus using a medical bone marrow drill and the possibility of subsequently injecting cream into the sinuses, with comparison to the classic surgical trephination. Using computed tomography (CT), we evaluated the degree to which the frontal sinuses and the nasal cavity were filled by the cream, as well as assessing for iatrogenic damage by any of these techniques. Our hypothesis was that the medical drill would be as fast as the surgical trephination of the frontal sinuses and equally efficient for injecting the cream.

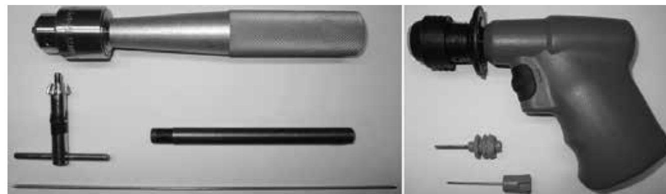

Figure 1. Intramedullary pin in a Jacob chuck (left) and medical drill (Arrow OnControl Powered Bone Access System) with a set of needles (right).

Materials and methods

Eleven heads from canine cadavers were collected and kept frozen until use. We used only dogs that weighed from 20 to 50 kg, had no evidence of facial trauma or nasal disease, and had a mesencephalic or dolichocephalic conformation. All dogs had died or were euthanized for reasons other than disease of the nasal cavity. Cadavers were excluded from the study if there was evidence of lesions affecting the nasal cavity or frontal sinuses on the initial CT-scan so that results could be compared among a homogenous population of dogs.

All heads were thawed over a period of 48 h before use. An initial CT-scan of the head ruled out any structural defects that would interfere with or complicate trephination of the frontal sinus. The CT-scan used in this study was a Toshiba Asteion 16 (Toshiba, Tustin, California, USA). Both trephination methods were carried out on each head, medical trephination on 1 side and surgical trephination on the other side.

A random-number generator was used to determine the order of the trephination procedures (surgical *versus* medical) and to determine, for the first head, whether the left or the right frontal sinus was trephined first. The order of the trephination procedure was alternated for all subsequent heads, with the left sinus always trephined first. Both sides of the head were prepared for aseptic trephination. Operators wore sterile gloves for all procedures. After the initial CT-scan of the head, the 2 frontal sinuses were trephined in the pre-established order. For both techniques, an incision was made through the skin and soft tissues over the frontal sinuses midway between the zygomatic processes of the frontal bone and the midline.

A board-certified surgeon (SS) carried out the surgical trephination procedure. A 1-cm skin incision was made over the frontal sinus. Then a 5/64″ intramedullary pin in a Jacobs chuck (Jacobs Chuck, Sparks, Maryland, USA) (Figure 1) was used to drill a hole manually in the frontal bone into the frontal sinus. After insertion, the pin was removed and a 5Fr polyethylene catheter (MILA, Florence, Kentucky, USA) cut to 2.5 cm was then introduced through the trephine hole to allow injection of the flush solution followed by the clotrimazole cream.

For the purpose of the study, in order to provide better contrast with adjacent soft tissue on the post-procedure CT-scan, the clotrimazole cream was prepared using 95% of 1% clotrimazole cream (Clotrimaderm; Taro Pharmaceuticals, Bramalea, Ontario) and 5%

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Table I. Scoring system for filling of nasal cavity

Filling of nasal cavity	76% to 100%	50% to 75%	0% to 50%
Score	Very good	Good	Poor

of 52% iodinated product (Omnipaque; GE Healthcare, Mississauga, Ontario) and kept at room temperature.

An inexperienced operator (VC) carried out the medical technique. A 1-cm skin incision was made over the frontal sinus. A medical bone marrow drill (Arrow OnControl Powered Bone Access System; Teleflex, Jaffrey, New Hampshire, USA) connected to a 15-gauge 25-mm bone marrow aspiration needle (Figure 1) was then used to trephine the sinus. The drill was then removed with the bone marrow needle left in place to allow the flush solution to be injected, followed by the clotrimazole cream.

The length of time for the surgical procedure was recorded from the time of initial skin incision to when the catheter was inserted through the trephine hole in the bone (A). For the medical procedure, timing A was recorded from the time of initial skin incision to when the intraosseous catheter was seated into the bone with the tip within the frontal sinus and the drill disengaged from it. After trephination, 120 mL of saline was simultaneously injected through the previously positioned catheters by both operators in both sinuses, using 2 preloaded 60-mL syringes. Saline solution was injected to ensure patency of the nasofrontal ostium and remove debris before injecting the cream, to simulate what would be done in a clinical setting. The time required to inject the saline was recorded for each operator (B). Following this, both operators injected 20 mL of the previously mixed clotrimazole cream into both sinuses simultaneously. Time required for each operator to inject the cream was recorded (C). The quantity of cream was extrapolated from previous studies that demonstrated good filling of the frontal sinuses with this volume (13,15). To our knowledge, there is no consensus on the quantity of cream to inject into the frontal sinuses. An independent observer who was not involved in either of the procedures timed all components of the procedure. The total time for each procedure was the sum of these 3 components (A + B + C).

A second CT-scan was carried out within 5 min after the clotrimazole cream was injected. The pre- and post-treatment images were analyzed using both bone and soft tissue windows to highlight bony detail and the degree of fill of the clotrimazole cream within the frontal sinuses, the nasal cavities, the common nasopharynx, and the laryngopharynx. The images were reviewed by a board-certified radiologist (GS) for completeness of filling of the sinuses, nasal cavities, nasopharynx, and larynx and for assessment of any evidence of trauma associated with the procedure. The radiologist was blinded to the trephination procedure carried out on each sinus. The head remained in ventral recumbency for the whole procedure.

The total volume of both frontal sinuses was measured with a diagnostic imaging software (OsiriX) using the initial CT scan images, based on 3-dimensional (3-D) reconstruction of the frontal sinuses or the cream. The volume occupied by the cream was measured by the same technique using the post-injection CT scan images. The percentage of filling of the sinus was then calculated by dividing the volume occupied by the cream by the total volume of the sinus. A subjective assessment of the filling of nasal cavities was recorded using the scoring system outlined in Table I. Qualitative assessment of the presence or absence of cream in the nasopharynx, larynx, maxillary sinuses, and sphenoid sinuses was also recorded for each head.

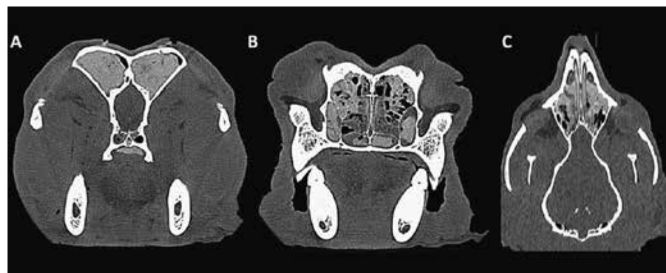

Figure 2. Computed tomographic images: Axial view of the frontal sinuses filled with cream (A), axial view of the nasal cavity filled with cream (B), and dorsal multi-planar reconstruction view of the nasal cavity filled with cream (C). Note the difference in density between the cream and the soft tissue.

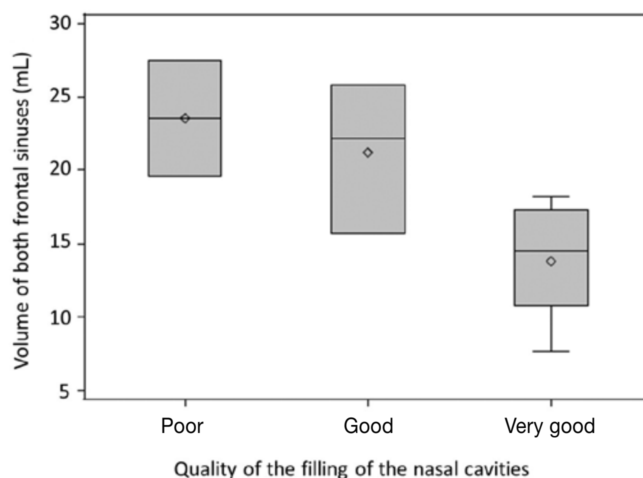

Figure 3. Quality of the filling of the nasal cavity related to the volume of both the frontal sinuses.

A statistical analysis was done using a Wilcoxon signed-rank test to compare the 2 trephination methods in terms of time required, the difference in the fill volume of the right and left sinus in each dog, and the difference in the amount of sinus fill by the cream. A Kruskal-Wallis test was used to assess whether there was a relationship between the volume of both frontal sinuses and the degree of nasal cavity filling by the cream. A Wilcoxon rank-sum test was used to evaluate the correlation between the volume of the frontal sinuses and the presence of cream in the nasopharynx, larynx, sphenoid sinuses, or maxillary sinuses.

Results

All 11 dogs used in this study had mesencephalic conformation, weighed from 20 to 50 kg, and were mixed breeds. None had any evidence of nasal or sinus disease detected on the initial CT-scan. The mean volume of the sinuses was 8.9 mL (3.1 to 13.2 mL) for the left sinus and 8.6 mL (4.5 to 14.3 mL) for the right sinus. There was no significant difference between the volume of the left and right sinuses in each dog ($P = 0.24$).

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The mean duration for the initial catheter insertion (A) was 28 s (13 to 66 s) for the surgical procedure and 17 s (11 to 30 s) for the medical drilling procedure. The mean duration for the saline flushing (B) was 17 s (9 to 24 s) and 14 s (12 to 20 s) for the surgical and medical procedures respectively and the mean duration of the injection of the clotrimazole cream (C) was 20 s (6 to 40 s) for the surgical procedure and 17 s (10 to 20 s) for the medical procedure. The overall duration of the procedure (A + B + C) was 47 s (34 to 68 s) for the medical drill and 67 s (39 to 116 s) for the surgical trephination. The trephination (A) and the overall procedure (A + B + C) were significantly faster using the medical drill than the surgical drill ($P = 0.001$ and $P = 0.006$, respectively). There was no significant difference in the duration for saline flushing and clotrimazole cream injection.

Mean filling of the frontal sinus was 82.4% (77% to 86%) for the surgical procedure and 82.7% (67% to 98%) for the medical procedure (Figure 2). There was no significant difference in the percentage of filling between the 2 techniques (P-value = 0.3). All trephinations involved the rostral compartment of the frontal sinuses.

Filling of the nasal cavities was very good in 6 cases (Figure 2), good in 3 cases, and weak in 2 cases. The filling of the nasal cavities was significantly more likely to be classified as good if the volume of the frontal sinuses was smaller for a given quantity of cream (Figure 3). Cream was present in the nasopharynx in 6 cases and in the larynx in 2 cases. There was cream in the right maxillary sinus in 7 cases, in the left maxillary sinus in 5 cases, and in both in 3 cases. There was cream in both sphenoid sinuses in 6 cases and only in the right sphenoid sinus in another case. There was no significant correlation between the volume of the frontal sinuses and the presence of the cream in these locations.

One cadaver head had an iatrogenic lesion with the surgical trephination and damage to the ipsilateral maxillary sinus caused by the pin was present on the second CT-scan.

Discussion

To summarize, the medical procedure was highly feasible even when carried out by an inexperienced operator and significantly faster than the surgical procedure by an experienced operator. It allowed the cream to be injected efficiently and did not cause any iatrogenic lesions.

The volume of the frontal sinuses varied widely among the cadaver heads used in this study (3.1 to 14.3 mL). This discrepancy, which is consistent with what has been described in a previous study (11), is probably related to the weight of the dogs, which was unfortunately not precisely known in this study, even though all heads were selected from dogs that weighed from 20 to 50 kg. A larger study and ideally in live dogs would be useful to help develop an equation or a chart linking these 2 parameters. This would allow the volume of the sinuses to be approximated from the weight of the dog for a given skull conformation, e.g., mesencephalic.

Based on our findings, the degree of filling of the nasal cavities is highly correlated to the total volume of the frontal sinuses for a given quantity of cream. Knowing the volume of the frontal sinuses (by measurement on the preliminary CT-scan or approximation using the weight of the dog) could allow the optimal quantity of cream to fill both the frontal sinuses and the nasal cavity to be determined in a given dog. This seems important clinically as failure to extensively fill the front sinuses and nasal cavity could potentially result in the treatment failing due to inadequate mucosal exposure.

Moreover, the nasopharynx should be packed in a live dog, which would prevent leakage in the nasopharynx and therefore limit the potential complication of using an excessive volume of cream and probably improve diffusion in the nasal cavities. In this study, the nasopharynx was not packed because of the difficulty this would have posed in the cadaver heads used.

Since both techniques were carried out in the same dog, it is impossible to compare the degree of filling of the nasal cavities, nasopharynx, and larynx between the 2 techniques, as we do not know if the cream can spread from 1 sinus to the contralateral nasal cavity. Given the similarities of the observations in the frontal sinuses, however, it can be extrapolated that both procedures would induce the same pattern of diffusion of the cream in the upper airways in a given dog.

We reached a very good degree of filling of the frontal sinuses with both techniques: 82.4% (77% to 86%) for the surgical trephination and 82.7% (67% to 98%) for the medical drilling procedure. The results for the extent of cream filling are comparable to previous studies, in which it was assessed by either measuring areas on 1 (15) or several (14) transversal CT-scan slices or using a subjective assessment (16). As we used volumes, it is difficult to compare our results with previous studies, but we believe that computing the actual volume of the sinus and the cream in the sinus better represents the true filling of the cavity. Indeed, if repartition of the cream is not homogenous or if the sinus is particularly irregular in shape, relying on the volume would be more representative.

It is interesting that a good filling of all 3 compartments of the frontal sinuses (rostral, medial, and lateral) was reached even though only 1 compartment was trephined and the septae between compartments remained intact. It therefore seems possible to appropriately treat every compartment by trephinating a single site, which is in agreement with previous studies (12,15).

There was no significant difference between the 2 procedures in the degree of filling of the frontal sinuses with the cream. The medical procedure was significantly faster than the surgical one, however, even though it was carried out by an operator with very little experience in frontal sinus trephination. Therefore, when it is not necessary to visualize the sinus by fibroscopy or debridement, the medical drill can be recommended for instilling clotrimazole cream in the canine frontal sinuses. If visualization of the frontal sinus before treatment was indicated, then a surgical trephination with a larger diameter bone pin would be the preferred technique. This would accommodate a small diameter fiberoptic or video endoscope being inserted for visualization of the sinus cavity and endostial lining. Sinusoscopy may be warranted in cases of aspergillosis when the disease is highly suspected but no detectable fungal plaques are detected in the nasal cavity (17) or when the CT-scan confirms obvious sinus involvement and debridement of fungal plaques is deemed appropriate before to instill antifungal cream. These cases likely represent a limitation to the use of medical drilling for trephination of the frontal sinuses.

The length of time for trephination procedures described in the literature for aspergillosis treatment remains quite long, especially when debridement is indicated, ranging from a mean duration of

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176 min with use of both solution and cream to 73 min for injecting the cream alone (13). Shorter procedures have been described with 5 min infusion of the antifungal solution, followed immediately by injection of the antifungal cream without debridement, with a total duration of 31.7 min (12). When carried out on a cadaver, the average length of our procedure was 47 s (38 to 68 s) with the medical drill. Injecting the cream alone, without infusion of the solution, is likely to be as fast or faster than the shortest previously described protocols, even when accounting for the time required for anesthesia and closure of the 1-cm skin incision.

The absence of iatrogenic lesions after the trephination procedure using the medical drill, even when carried out by an inexperienced operator, suggests that this is a relatively safe technique. The surgical trephination was associated with damage to a maxillary sinus in a single case. The clinical significance of such a lesion is likely to be negligible and it was not associated with any reduction in the degree of filling of the sinus. A study on approximately the same number of dogs trephined surgically reported complications in 3/12 cases, with infusion of the cream around the brain and in the periorbital tissue (15). The fact that the dogs were smaller in that study might explain the higher number of complications. In this study as in ours, the surgeon was not aware of the trauma during the trephination, which could justify a post-trephination CT-scan, besides assessing diffusion of the cream.

The inconsistent filling of the maxillary sinus seen in this study could represent a potential problem for resolving aspergillosis infection as well as possibly causing a relapse. The presence of lesions in this location is rarely described (18), however, and it has been proven that the cream persists in the frontal sinus for a least 96 h in cadavers (11), which could still allow the cream to act on the maxillary sinus by diffusion. It would be ideal to conduct this procedure in established cases to see how long the cream persists within the sinus in a live dog with aspergillosis.

Cheaper medical drills could potentially be used in order to trephine the frontal sinuses, including some developed by the same company as the drill used here. Further studies would be necessary to determine whether the same results would be achieved using other medical drills. These drills can also be used for other purposes, e.g., fluid resuscitation via osseous catheter and bone marrow aspiration.

The use of dogs without nasal disease can be looked at as a limitation in our study. The distribution of the cream could be affected by turbinate or bone destruction caused by aspergillus infection or any other disease. The use of cadavers instead of live dogs is another limitation, since diffusion of the cream is likely to be different in living animals and we could not assess complications such as subcutaneous emphysema or infection. Not knowing the precise weight of the dogs in this study is an important limitation, as it was not possible to determine if there was a correlation between the dog's weight and the sinus volume, with the latter having a direct impact on the diffusion of the clotrimazole cream in our study. Nevertheless, dogs used in this study all weighed more than 20 kg, which represents the size of dog typically affected by aspergillosis.

We believe that the use of a medical drill designed for bone marrow aspiration can be an advantageous alternative to surgical trephination of the frontal sinuses in dogs with aspergillosis, when debridement or sinusoscopy is not deemed necessary. When injection of cream into the frontal sinuses or nasal cavity is warranted, calculation of the volume of the sinuses before trephination using a 3-D reconstruction of CT-scan images might allow a better estimation of the volume of cream to be injected.

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Escherichia coli isolated from feces of brown bears *(Ursus arctos)* have a lower prevalence of human extraintestinal pathogenic *E. coli* virulence-associated genes

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Abstract

Eighty-six *Escherichia coli* strains from feces of either wild brown bears or those living in a zoo were screened for phylogenetic groups using the revisited Clermont phylotyping method and the prevalence of 24 virulence-associated genes (VAGs) of extraintestinal pathogenic *E. coli* (ExPEC). Our results showed that most strains of *E. coli* in bears belonged to phylogenetic groups III/IV/V (29%) and B1 (26%). Only half of the tested VAGs were found in the *E. coli* bear strains, with *fimH* present in 72%, *ompT* in 63%, and *kpsMT* in 43% of the strains. When the data obtained on the fecal *E. coli* strains from brown bears were compared with the data obtained on 90 fecal *E. coli* strains from healthy humans, there were significant differences in *E. coli* population structures between both hosts.

Résumé

*Quatre-vingt-six souches d'*Escherichia coli *provenant de fèces d'ours brun vivant en nature ou dans un zoo ont été analysées pour déterminer les groupes phylogénétiques à l'aide de la méthode de phylotypage Clermont révisée et la prévalence de 24 gènes associés à la virulence (GAVs) d'*E. coli *pathogène extra-intestinal (ExPEC). Nos résultats ont montré que la plupart des souches d'*E. coli *chez les ours appartenaient aux groupes phylogénétiques III/IV/V (29 %) et B1 (26 %). Seulement la moitié des GAVs testés ont été trouvés dans les souches d'*E. coli *d'ours,* fimH *étant présent chez 72 %,* ompT *chez 63 %, et* kpsMT *chex 43 % de ces souches. Lorsque les résultats des souches d'*E. coli *obtenues des matières fécales d'ours brun ont été comparés aux données obtenues à partir de 90 souches fécales d'*E. coli *d'humains en santé, il n'y avait aucune différence significative dans la structure des populations d'*E. coli *entre les deux hôtes.*

(Traduit par Docteur Serge Messier)

Mammals have a complex gut microbiota that is shaped by intestinal anatomy, function, and diet (1). *Escherichia coli* is part of the normal intestinal microbiota and coexists with its host in mutual benefit. The intestinal microbiota can also be a reservoir of extraintestinal pathogenic *E. coli* (ExPEC) and it is known that animals, e.g., cats, dogs, and birds, can be a potential reservoir of ExPEC (2). Although extraintestinal infections due to *E. coli* are not known to be a significant issue in bears, some reports link *E. coli* with extraintestinal infections. For example, *E. coli* was isolated from cellulitis of an American black bear *(Ursus americanus)* (3) and *E. coli* septicemia was found in neonatal Polar bears *(Ursus maritimus)* (4).

The pathogenic potential of *E. coli* is closely related to the presence of virulence-associated genes (VAGs), such as adhesins, capsules, iron uptake systems, invasins, and toxins (5). While the brown bear *(Ursus arctos)* is the most widely distributed of bears, it is nevertheless an endangered species about which we lack data. As there are no data on *E. coli* strains from the intestinal microbiota of the brown bear, the aim of this study was to investigate and characterize fecal *E. coli* from brown bears for phylogenetic group and most typical ExPEC VAGs.

The phylogenetic group of the studied strains was determined using the revisited Clermont method, which is a novel quadruplex polymerase chain reaction (PCR) with a higher rate of correct classification (over 95%) than the traditional Clermont method (6). In addition, the sequence types (ST) of the B2 phylogenetic group strains were identified. Analysis with PCR was also used to determine the prevalence of VAGs among the studied *E. coli* strains. As composition of the intestinal microbiota is affected by the diet and environment of the host (7), we also compared *E. coli* strains from wild bears with bears housed in a zoo. Furthermore, the obtained data were compared with data gathered on 90 fecal *E. coli* strains from healthy humans to gain insight into host adaptation to *E. coli.*

We investigated a collection of 86 *E. coli* strains isolated from the feces of brown bears. Forty-five strains were isolated from the feces of wild brown bears located in the forest region of Kočevje, Slovenia and 41 strains were from 3 captive adult brown bears housed at the Ljubljana Zoo in Slovenia. The studied *E. coli* strains were isolated from 18 different samples of brown bear feces that were collected from October 2010 to April 2012. Four fecal samples were from brown bears in the zoo and 14 were from brown bears living in the

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Table I. Comparison of distribution of phylogenetic groups among fecal *E. coli* strains from brown bears and from humans determined using the revisited quadruplex Clermont method

	Prevalence (% of the tested strains)		
Phylogenetic group	Bears (86 strains)	Humans (90 strains)	*P-value**
A	13	13	
B1	26	11	
B2	6	33	0.00003
C	0	1	
D	3	8	
E	15	21	
F	6	7	
I/II	1	0	
III/IV/V	29	2	3.3×10^{-6}
Unknown	1	3	

* *P*-values were calculated and only those that were statistically significant are given.

wild. The *E. coli* strains were initially isolated as lactose-positive colonies on MacConkey agar plates. Subsequently, growth was monitored on eosin methylene blue (EMB), as well as Uriselect plates and the indole test. Enterobacterial repetitive intergenic concensus-polymerase chain reaction (ERIC-PCR) was carried out to ascertain that only distinct *E. coli* strains were included in the study (8). The investigated strains were cultivated in Luria Bertani medium or agar.

Ninety *E. coli* strains isolated from the feces of healthy human volunteers, which were collected and partially characterized in a previous report (9), were also included in the present study. Bacterial deoxyribonucleic acid (DNA) used for PCR analyses was extracted according to standard protocols. All strains were assigned to phylogenetic groups A, B1, B2, C, D, E, F, I/II, III/IV/V, or unknown, using the revisited quadruplex Clermont method (6). The amplified DNA fragments were visualized by agarose gel electrophoresis using ethidium bromide staining. Amplicons were photographed with UV exposure and their lengths verified by a DNA ladder standard. The phylotyping was done in duplicates.

Escherichia coli bear strains assigned to the B2 phylogenetic group were additionally characterized by multilocus sequence typing (MLST), which was carried out as previously described by Wirth et al (10). Nucleotide sequences of genes *adk, fumC, gyrB, icd, mdh, purA,* and *recA* were amplified with primers and PCR conditions listed in Supplementary Table I and the primers and PCR conditions used to amplify VAGs are summarized in Supplementary Table II (both available at: http://www.bf.uni-lj.si/fileadmin/users/1/biologija/genetika/E-coli-from-brown-bears-Supplementary-Tables.pdf). Deoxyribonucleic acid (DNA) sequencing of the purified PCR products was conducted by Macrogen (South Korea). The obtained sequences were compared with known alleles and sequence types (STs) were assigned using MLST database for *E. coli* (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

The 24 analyzed ExPEC VAGs were: *ompT* (outer membrane protease T); APEC-*ompT* (avian pathogenic *E. coli* outer membrane protease T); *clbAQ* (genomic island that encodes the genotoxin colibactin); *traJ* (positive regulator of conjugation); *iss* (increased serum survival); *traT* (serum resistance); *neuB* (capsule K1, sialic acid Neu5Ac synthase); *kpsMT* (group II capsule); *papGII* (pyelonephritis-associated adhesin gene class II); *papGIII* (pyelonephritis-associated adhesin gene class III); *sfaDE* (S fimbrial adhesion); *afa/draBC* (adhesins of the AFA-DR family); *cnf1* (cytotoxic necrotizing factor); *hlyA* (hemolysin); *usp* (uropathogenic specific protein); *iucD, fyuA, ireA,* and *iroN* (siderophore receptors); *tcpC* (TIR homologous protein); *ibeA* (brain microvascular endothelial cells invasion); *fimH* (type 1 fimbriae); *iha* (iron-regulated gene A homologue adhesin); and *hbp* (hemoglobin protease).

Table II. Comparison of prevalence of virulence-associated genes (VAGs) among fecal *E. coli* strains from brown bears and from humans

	Prevalence [number (%) of the tested strains]		
	Bears (86 strains)	Humans (90 strains)	*P-value**
Virulence-associated genes (VAGs)			
Toxins			
cnf1	0	6	
hlyA	0	8	
usp	7	68	1.4×10^{-16}
ibeA	16	13	
Fimbriae and/or adhesins			
fimH	72	88	
papGII	0	8	
papGIII	0	3	
sfaDE	0	17	0.00081
afa/draBC	0	4	
Iron uptake			
iucD	2	39	7.9×10^{-9}
fyuA	21	66	4.7×10^{-8}
ireA	0	20	0.00008
iha	0	39	3.1×10^{-11}
hbp	0	8	
iroN	9	29	0.02606
Capsule			
kpsMT	43	58	
neuB	0	27	5.6×10^{-7}
Other			
tcpC	0	8	
APEC-*ompT*	2	22	0.00118
ompT	63	71	
clbAQ	0	16	0.00177
traJ	2	26	0.00013
iss	6	14	
traT	14	62	6.4×10^{-10}

* Only statistically significant *P*-values are given.

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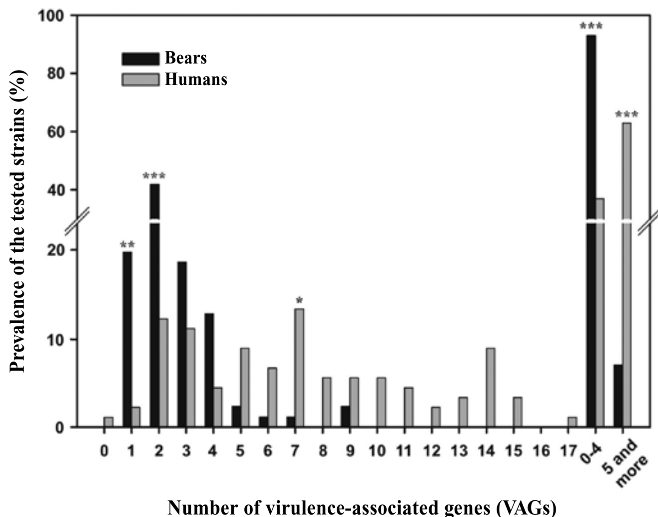

Figure 1. Comparison of VAG numbers among fecal *E. coli* strains from brown bears and human fecal *E. coli* strains. Only statistically significant *P*-values are given: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Polymerase chain reactions (PCR) were carried out in an automated thermal cycler (UNOII; Biometra, Göttingen, Germany) in 25 μL reaction mixtures containing 5 μL of template DNA, 20 pmol of forward and reverse primer, 0.2 mM 2′deoxynucleoside 5′-triphosphate (dNTP), 2.5 mM magnesium chloride ($MgCl_2$), 1× *Taq* buffer, 0.625 U *Taq* DNA polymerase, and distilled water to 25 μL (Fermentas, Vilnius, Lithuania). All the PCR reactions were done in duplicate. Prevalence of VAGs in strains from bears and healthy humans was analyzed using Fisher's exact test (2-tailed) (http://www.langsrud.com/fisher.htm). Due to multiple correlations, the Bonferroni correction was applied. The threshold for statistical significance after Bonferroni correction was set at *P*-values of < 0.05.

All 86 studied *E. coli* bear strains and 90 human strains were assigned to phylogenetic groups using the revisited Clermont phylotyping PCR method (6). The obtained data are presented in Table I. With the revisited Clermont phylotyping method, 59 (69%) of the studied brown bear strains could be assigned to a phylogenetic group (A to F), while the remaining 27 strains (31%) belonged to cryptic or unknown clades (I to V). The revisited Clermont method revealed that strains of the B1 phylogenetic group were the most prevalent, with 22 strains (26%), followed by group E with 13 strains (15%), and group A with 11 strains (13%). However, among the investigated human strains, the largest phylogenetic group was B2 with 30 strains (33%). The difference in the prevalence of B2 strains in brown bears and in humans was statistically significant. Furthermore, strains of the cryptic clades III/IV/V were significantly more prevalent in brown bears than in humans (Table I).

The 5 B2 strains were further analyzed for sequence types (STs) using MLST. Three strains belonged to ST174, 1 to ST1459, while 1 strain had a new combination of alleles. The strain with a new ST type had the following combination of alleles: ADK175 — FUMC356, a GYRB that most resembles GYRB301 with 459/460 matches (on the position 357 is a T, while in GYRB301 is a C), ICD304 — MDH242, a PURA that most resembles PURA282 with 477/478 matches (on the position 329 is a G, while in the PURA282 is a T),

Table III. Comparison of distribution of phylogenetic groups and virulence-associated genes (VAGs) among fecal *E. coli* strains from brown bears in zoo and in the wild

	Prevalence [number (%) of the tested strains]		
Trait	Zoo (41 strains)	In the wild (45 strains)	*P-value**
Phylogenetic group			
A	24	2	0.02606
B1	7	42	0.00191
B2	10	2	
C	0	0	
D	0	7	
E	17	13	
F	12	0	
I/II	2	0	
III/IV/V	27	31	
Unknown	0	2	
Virulence-associated genes (VAGs)			
Toxins			
cnf1	0	0	
hlyA	0	0	
usp	12	2	
ibeA	20	13	
Fimbriae and/or adhesins			
fimH	73	71	
papGII	0	0	
papGIII	0	0	
sfaDE	0	0	
afa/draBC	0	0	
Iron uptake			
iucD	0	4	
fyuA	29	13	
ireA	0	0	
iha	0	0	
hbp	0	0	
iroN	0	18	
Capsule			
kpsMT	44	42	
neuB	0	0	
Other			
tcpC	0	0	
APEC-*ompT*	0	4	
ompT	71	56	
clbAQ	0	0	
traJ	0	4	
iss	0	11	
traT	2	24	

* Only statistically significant *P*-values are given.

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and a RECA that most resembles RECA130 with 508/510 matches (on the position 361 is a C, while in the RECA130 is a T, and on the position 365 is a C, while in the RECA130 is a T).

The studied *E. coli* strains were screened for the prevalence of 24 VAGs commonly found among ExPEC strains (5). The obtained data are summarized in Table II. Among *E. coli* strains isolated from brown bears, 12 different VAGs were detected. The most prevalent were *fimH* in 62 strains (72%), *ompT* in 54 (63%), *kpsMT* in 37 (43%), *fyuA* in 18 (21%), and *ibeA* in 14 strains (16%). While the presence of all tested VAGs was ascertained among the *E. coli* strains isolated from humans, the 5 most prevalent were *fimH* (88%), *ompT* (71%), *usp* (68%), *fyuA* (66%), and *traT* (62%).

Comparison of VAG prevalence of *E. coli* strains between brown bears and humans showed that 12 out of 24 VAGs (*usp, sfaDE, iucD, fyuA, ireA, iha, iroN, neuB,* APEC-*ompT, clbAQ, traJ,* and *traT*) were found to be statistically significant less often among *E. coli* strains from brown bears than among those from healthy humans (Table II). Analysis of the number of VAGs present in fecal bear and human *E. coli* revealed that there were fewer statistically significant strains with 5 or more VAGs in bear strains than in human strains (Figure 1). This difference was also reflected in a lower average virulence score among brown bear strains than human fecal *E. coli* strains, 2.6 *versus* 7.2, respectively (Figure 1).

Comparison of *E. coli* strains obtained from brown bears in the wild and those living in a zoo showed that strains belonging to the phylogenetic group A were statistically significantly associated with captive bears and group B1 strains were associated with wild bears ($P < 0.05$). No major difference was detected in the prevalence of the tested VAGs in both bear populations (Table III).

A number of studies have explored the characteristics of *E. coli* strains from different animal hosts and their relevance for virulence potential (2). While previous studies have defined the fecal microbiota and intestinal metabolic activity of polar and grizzly bears (7,11,12), to our knowledge there is no data on molecular characterization of *E. coli* strains from the endangered brown bear. In the present study, we therefore focused on the phylogenetic distribution and ExPEC VAGs of *E. coli* strains from captive and free-roaming brown bears. Due to genetic diversity, *E. coli* strains may be assigned to phylogenetic groups. The first reliable PCR method enabled assignment to 1 of 4 phylo-groups: A, B1, B2, or D (13). In 2013, a new quadruplex PCR method was introduced (6) that recognized groups A, B1, B2, C, D, E, F, and cryptic clades I to V. Only the new method was used in our study, as it was identified as being more reliable (6).

In their study of predominant aerobic microbiota of black bears *(Ursus americanus)* and grizzly bears *(Ursus arctos),* Goatcher et al (14) showed that the microorganisms isolated from bear samples could also be found in plant, water, and soil samples from the study area. This indicates that the predominant bear microbiota were influenced by the foraging habits and surrounding environments of the bears. Brown bears consume a predominantly vegetative diet (15), although they can also consume meat and dairy products. Hunters may provide bears with carcasses of wild animals at feeding stations in their natural environment. Bears can also come in contact with human food as they approach villages that are close to woods, orchards, and farmlands and by rummaging through garbage. As bears have an excellent sense of smell, they also easily find food discarded by humans visiting the woods. Compared to bears in the wild, brown bears living in captivity are in closer contact with humans through caretakers who clean their cages and handle their food as well as visitors who sometimes throw food. The bears' drinking water can also be a source of bacteria. Bears in the wild generally use several water sources, while a natural pond in their enclosure is the only source of water for bears at the Ljubljana Zoo.

The differences in food and water supplies among bears living in the wild and those in captivity could affect the prevalence of *E. coli* phylogenetic groups. In general, in this study, the phylogenetic distribution of strains from wild and captive bears was similar, except that group A was statistically significantly associated with captive bears and group B1 was associated with wild bears ($P < 0.05$). These results correlate with the known fact that group A strains are more prevalent in carnivores and omnivores than in herbivores, while in herbivores, most *E. coli* strains belong to group B1 (16). Of the 5 B2 strains, 4 were isolated from captive bears. This is not unexpected as there is closer contact between bears and humans at a zoo. Even though group B2 strains are associated with human extraintestinal *E. coli* infections, they are also prevalent among human fecal microbiota (17). Multilocus sequence typing (MLST) analysis of the studied B2 strains in bears showed that 3 strains belonged to ST174. This ST was recently determined for a B2 porcine fecal strain with resistance genes against 7 antibiotics (amikacin, chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin, cotrimoxazole, and tetracycline) (18), which indicates a high potential for adapting to different environments.

To further our understanding of *E. coli* strains from brown bears, we screened our strain collection for the prevalence of several extraintestinal VAGs. A high prevalence of VAGs *fimH, ompT, kpsMT,* and *fyuA* was detected, which again indicates a virulence potential, albeit low, among the examined *E. coli* strains from captive and wild brown bears.

The gathered data on phylogenetic groups and VAG prevalences among the studied bear fecal *E. coli* strains were compared to data on phylogenetic groups and VAG prevalences among human fecal *E. coli* strains. A significant difference was revealed in the phylogenetic structure of both *E. coli* populations. Among the brown bear fecal *E. coli* strains, there were less statistically significant B2 strains and more III/IV/V strains than in human fecal *E. coli* strains. A lower prevalence of VAGs was revealed in bear strains compared to human strains. Bear strains also have a lower number of VAGs per strain than in human strains. The obtained data clearly showed a significant difference in the genetic structure of the *E. coli* from the 2 hosts, bears and humans.

Virulence factors encoded in VAGs associated with ExPEC could represent fitness factors involved in intestinal colonization and survival among the B2 group strains (19). Ursids are characterized by a simple gut physiology with rapid digestion (1) that could eliminate the need for fitness factors and explain the low prevalence of the B2 group, as well as a general low prevalence of VAGs among the examined *E. coli* isolates from brown bears. Iron uptake systems, such as siderophores, are a good example of the fitness factor nature of the virulence factors encoded in the VAGs, as iron is essential for bacterial growth. In this study, we examined

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the prevalence of 6 VAGs involved in iron acquisition. All 6 of the tested iron-acquisition systems were statistically significantly linked to human strains, while bear strains harbored only *fyuA* [prevalence of 18 (21%)], *iroN* [prevalence of 8 (9%)], and *iucD* [prevalence of 2 (2%)]. As this may be due to the different gut complexities of different hosts, our results support the idea that prevalence of different VAGs may reflect adaptation to commensal lifestyle in different hosts and ExPEC strains are only a by-product of commensalism (20). Further characterization of *E. coli* bear strains will contribute to a deeper understanding of the complex dynamics between *E. coli* and its host.

Acknowledgments

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Nasopharyngeal temperature measurement in sheep during general anesthesia

Tabita Tan, Jonathon Tuke, Gabrielle C. Musk

Abstract

The aim of this study was to compare nasopharyngeal and esophageal temperature measurements in anesthetized sheep with a range of fresh gas flows (1 to 6 L/min) through the breathing system. Data were compared using a Bland-Altman plot and correlation coefficients, and error measures were calculated. One hundred and ninety-five sets of data were collected from 20 sheep weighing 41 kg (31 to 51.5 kg). The bias (95% limit of agreement), correlation coefficient, and absolute error for nasopharyngeal compared to esophageal temperature were 0.04°C (−0.77°C to 0.85°C), 0.92, and 0.29°C ± 0.29°C, respectively. The percentage of nasopharyngeal readings within 0.5°C of the esophageal temperature was 77.44%. The error did not significantly increase with increasing fresh gas flow. Nasopharyngeal temperature measurement is suitable for estimation of esophageal temperature during general anesthesia of sheep when the fresh gas flow through the breathing system is between 1 and 6 L/min.

Résumé

L'objectif de la présente étude était de comparer les mesures des températures nasopharyngiennes et œsophagiennes chez des moutons anesthésiés avec une variation du flux de gaz frais (1 à 6 L/min) à travers le système respiratoire. Les données ont été comparées à l'aide d'un graphique de Bland-Altman et des coefficients de corrélation, et les erreurs de mesure ont été calculées. Cent quatre-vingt-quinze paires de données ont été obtenues de 20 moutons pesant en moyenne 41 kg (31 à 51,5 kg). Le biais (limite d'accord de 95 %), le coefficient de corrélation, et l'erreur absolue de la température nasopharngienne comparée à la température œsophagienne étaient 0,04 °C (−0,77 °C à 0,85 °C), 0,92, et 0,29 °C ± 0,29 °C, respectivement. Le pourcentage de lecture de température nasopharyngienne à l'intérieur d'un écart de 0,5 °C de la température œsophagienne était de 77,44 %. L'erreur n'a pas augmenté de manière significative avec l'augmentation du flux de gaz frais. La mesure de la température nasopharyngienne est appropriée pour estimer la température œsophagienne lors de l'anesthésie générale de moutons lorsque le flux de gaz frais à travers le système respiratoire se situe entre 1 et 6 L/min.

(Traduit par Docteur Serge Messier)

During general anesthesia, the normal thermoregulatory control of core body temperature in animals is impaired (1). Consequently, hypothermia is a common complication of general anesthesia, leading to prolonged anesthetic recovery times, bradycardia, post-operative wound infection, and coagulopathies (2). The degree of hypothermia experienced by the patient during general anesthesia is influenced by the duration of anesthesia, body condition, body position, and invasiveness of the procedure (3).

Body temperature is routinely monitored during general anesthesia for the maintenance of normothermia. However body temperature is not homogeneous within an individual (4) and can differ between the core (deep thoracic, abdominal, and central nervous system) and periphery (skin, arms, and legs) by a range of 2°C to 4°C in humans (1). Measurement of core temperature is the best indicator of thermal status in humans as it is usually tightly regulated by the body and changes in core temperature reflect a failure of effective thermoregulatory defenses rather than the effect of environmental factors. In contrast, the temperature of peripheral tissues may vary more markedly as a result of exposure to flat surfaces and the ambient temperature, combined with other physiological factors such as vasodilation (1).

Body temperature can be measured at various sites and ideally the method chosen is as representative of the core body temperature as possible. In animals, both esophageal and nasopharyngeal temperatures are considered acceptable ways to evaluate core temperature. When these sites are not easily accessible, rectal temperature can also be measured, but is not considered to be as accurate for estimation of the core body temperature (1).

Sheep are regularly used in biomedical research and frequently undergo general anesthesia as experimental subjects (5,6). It is recognized that despite having fleece, sheep can become hypothermic during general anesthesia if they are not actively warmed (7). Various sites for temperature measurement are accessible in sheep but have not been compared to each other. The purpose of this study was to investigate agreement between temperature measurements from the nasopharynx and esophagus. We hypothesized that the nasopharyngeal temperature would be an accurate site for measurement of core body temperature compared with the esophageal

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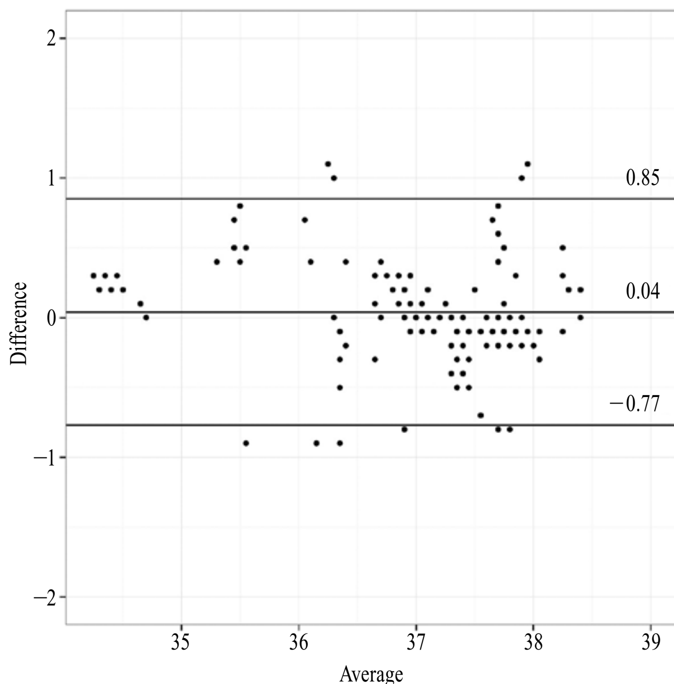

Figure 1. The bias for nasopharyngeal temperature compared to esophageal temperature.

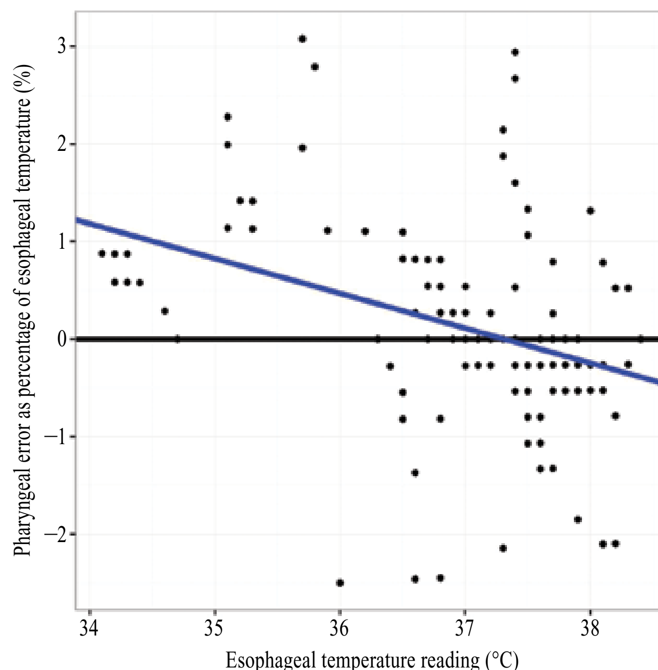

Figure 2. Correlation coefficient of pharyngeal temperature and esophageal temperature.

temperature. Furthermore we hypothesized that the fresh gas flow passing through the anesthetic breathing system would not affect the accuracy of nasopharyngeal temperature measurements.

This study was an opportunistic study undertaken on anesthetized sheep used in a project approved by the Animal Ethics Committee of the University of Western Australia according to the guidelines delineated in the National Health and Medical Research Council's Australian code for the care and use of animals for scientific purposes (8). The study population was a convenience sample of 20 adult dry merino cross ewes. The sheep were housed at the Large Animal Facility in raised individual pens for at least 1 wk prior to anesthesia. The holding rooms were temperature controlled between 20.5°C and 21.5°C and relative humidity was maintained between 40% and 60%.

Food was withheld for 12 h prior to anesthesia and on the day of anesthesia the sheep were weighed and pre-medicated with a combination of acepromazine (ACP 2 injection 2 mg/mL; Ceva Delvet, Asquith, New South Wales, Australia), 0.03 mg/kg body weight (BW) and buprenorphine (Temgesic 0.3 mg/mL; Reckitt Benckiser, West Ryde, New South Wales, Australia), 0.01 mg/kg BW by intramuscular injection. Anesthesia was induced 30 to 40 min later with a combination of midazolam (Midazolam 5 mg/mL; Pfizer Australia, West Ryde, New South Wales, Australia), 0.25 mg/kg BW and ketamine (Ketamil 100 mg/mL; Troy Laboratories, Smithfield, New South Wales, Australia), 5 mg/kg BW administered intravenously through a cannulated cephalic vein. Once anesthesia was induced, the trachea was intubated orally and anesthesia was maintained with isoflurane (1% to 2% Attane Isoflurane 1 mg/mL; Bayer Australia, Pymble, New South Wales, Australia) in 100% oxygen delivered through a circle breathing system. An experienced person adjusted the isoflurane vaporizer as necessary throughout the procedure, to maintain an adequate depth of anesthesia. The fresh gas flow was set at 1 L/min except when the temperature measurements were performed. Intermittent positive pressure ventilation was administered continuously with a tidal volume of 10 mL/kg and respiratory rate of 8 to 10 breaths/min to maintain the end-tidal CO_2 in the range of 40 to 55 mmHg. The sheep were in dorsal recumbency and were euthanized at the end of the procedure with intravenous pentobarbitone.

The sheep were monitored continuously with the aid of a Surgivet V9203 multivariable monitor (Smiths Medical, Rockland, Massachusetts, USA). The parameters measured by the Surgivet included oxyhemoglobin saturation, pulse rate, heart rate, respiratory rate, and end-tidal CO_2. Two Surgivet temperature probes were used for the temperature comparison. Throughout the study, each probe was nominated for use only in the esophagus or only in the nasopharynx and labeled as such. The probes were calibrated at the beginning of each session by simultaneous immersion into a water bath. Water temperature as recorded by the probes ranged from 22.1°C to 39.7°C and the difference in temperature between probes was a maximum of 0.7°C.

The esophageal temperature probe was passed through the mouth, into the esophagus and positioned so that its tip was at the level of the heart base. The nasopharyngeal temperature probe was passed through the nostril and situated so that its tip was resting at the level of the pharynx, near the internal carotid artery and adjacent to the soft palate. Both probes remained *in situ* for the duration of anesthesia. To ensure the probe was advanced far enough, a mark was placed on each probe to indicate the level of the incisors so the tip was at the medial canthus of the eye or the level of the caudal border of the scapula for the nasopharyngeal site and the esophageal site, respectively. Fresh gas flow was maintained at 1 L/min and

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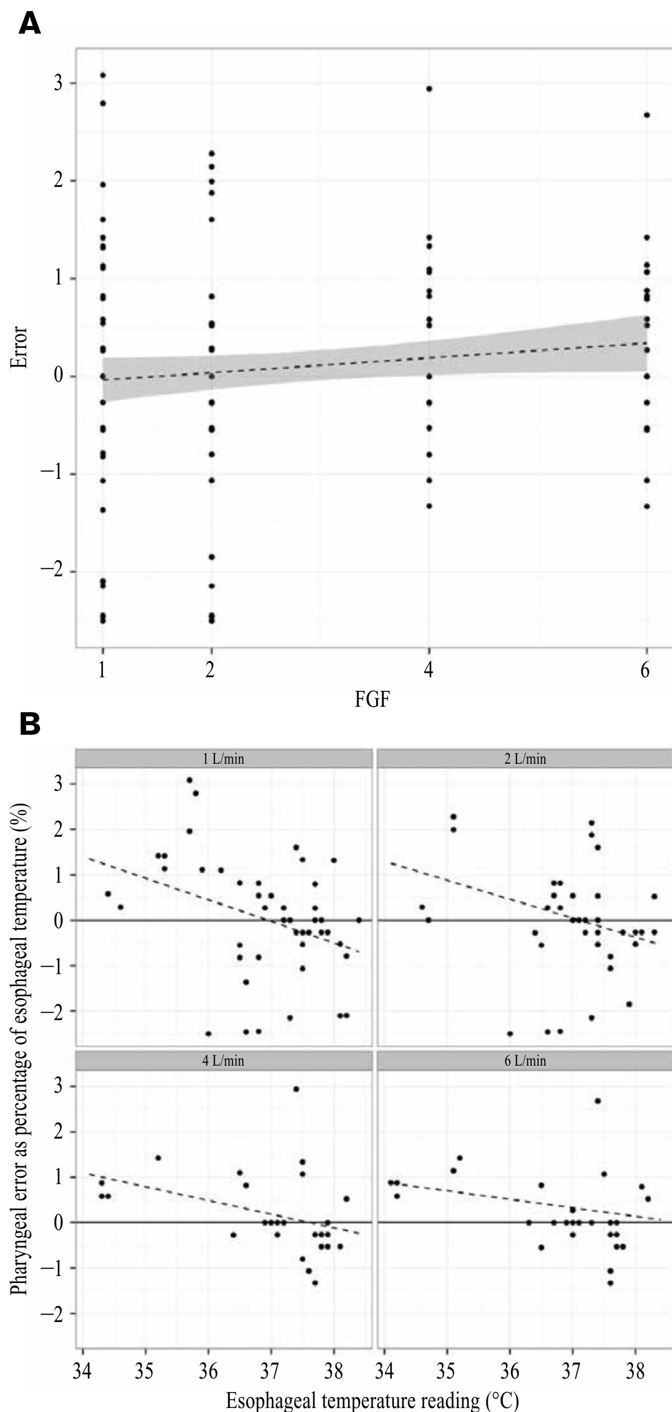

Figure 3. **A — Error between nasopharyngeal and esophageal temperature readings with increased gas flow. B — Effect of gas flow on the error of nasopharyngeal temperature compared with esophageal temperature measurement.**

altered 5 min prior to collecting paired temperature measurements at 2 L/min, 4 L/min, and 6 L/min. For these measurements the fresh gas flow was increased through the range of fresh gas flow starting at 1 L/min and increasing to 2 L/min, then 4 L/min, and finally 6 L/min.

Data were analyzed using the Bland-Altman method to calculate the bias and 95% limit of agreement using the MethComp package in R (R Foundation for Statistical Computing, Vienna, Austria). Correlation coefficients and error measures were also calculated and the impact of the fresh gas flow on the difference between the temperature measurements was determined. Data are expressed as median (range).

One hundred and ninety-five sets of data were collected from 20 sheep weighing 41kg (31 to 51.5 kg). The physiological parameters assessed during anesthesia were within normal limits throughout the study period. The oxyhemoglobin saturation was always greater than 95% and the pulse rate and heart rate were within the range usually observed in this setting. The end-tidal CO_2 remained in the target range. The body temperature was maintained in all sheep: esophageal temperatures were 37.3°C (34.1°C to 38.4°C), and nasopharyngeal temperatures were 37.2°C (34.4°C to 38.5°C).

The bias (95% limit of agreement) for nasopharyngeal temperature compared to esophageal temperature was 0.04°C (−0.77°C to 0.85°C) (Figure 1). The correlation coefficient and absolute error were 0.92°C and 0.29°C (± 0.29°C), respectively (Figure 2). The percentage of nasopharyngeal readings within 0.5°C of the esophageal temperature was 77.44%.

Although there was a slight increase in the error between nasopharyngeal and esophageal temperature readings as the fresh gas flow increased, the linear relationship was not significant. These results indicate that there was no effect of fresh gas flow on the error of nasopharyngeal temperature compared with esophageal temperature measurements (Figures 3a and 3b).

This study compared nasopharyngeal with esophageal temperature measurements in anesthetized sheep. The aim was to assess the agreement between measurements taken at these 2 sites when the fresh gas flow through the breathing system was changed. The nasopharyngeal site for temperature measurement accurately reflected the esophageal temperature and was not affected by the fresh gas flow through the breathing system in 41 kg (31 to 51.5 kg) merino ewes during general anesthesia. To our knowledge, no studies have been performed in sheep comparing these 2 non-invasive sites for temperature measurement.

Both nasopharyngeal and esophageal temperature measurements are considered to be reliable indications of core body temperature compared with rectal, bladder, and skin surface sites (1,9,10). This consistency is most likely greatest when the body temperature is stable. There is evidence that nasopharyngeal measurements adjust more slowly than esophageal measurements to changes in cerebral temperature (9,11) and that when profound hypothermia is rapidly induced and reversed, temperature measurements made at both these standard monitoring sites may not reflect cerebral temperature (10). The body temperature of the sheep in the current study was stable and normal so it is possible that the agreement between measurements at the 2 sites would have been diminished if the animal's temperature was more labile or out of the normal range.

The reliability of both the esophageal and nasopharyngeal probes as an indication of core body temperature depends on their correct placement. Probes that are not placed in the optimal position are more likely to be influenced by the artificial cooling effect of inspired gases (10). Esophageal temperature measurements are considered clinically useful to assess changes in core body temperature because the probe is easily introduced and there is a low risk of incorrect

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positioning. As such it is the preferred site for monitoring core body temperature (9). In this study, marks were made on the probes at the level of the incisors before introducing them into the animal, to reduce the risk of positioning the probes incorrectly at either site.

The reliability of the esophageal and nasopharyngeal temperature measurements as an accurate reflection of brain temperature depends on a number of factors including the ambient temperature, the rate of change in temperature, the local blood supply, the core body temperature, and the placement of the probe (10,12). Placing of the nasopharyngeal temperature probe was easier than placing the esophageal temperature probe in this study and given the results, it appears as though this site may be preferable and more convenient. However, Stone et al (10) suggest that when indirectly assessing brain temperature by less invasive means, multiple measurement sites should be used and that these methods should be in agreement to provide an accurate measure of true brain temperature. During general anesthesia of sheep for short procedures, it is routine clinical practice to measure body temperature from a single site, but for complex and prolonged procedures where body temperature may change, multiple sites for measurement may be appropriate.

The data in this study were analyzed in a number of ways to ensure that the results were robust. Bland-Altman analyses are commonly used to compare measurements at a novel site to an established one to determine whether the 2 methods can be used interchangeably (13). This analysis, along with the correlation coefficient and absolute error, demonstrate the accuracy of the nasopharyngeal site for temperature measurement. Furthermore, as it is considered unacceptable for the difference between temperature measurements at different sites to be greater than 0.5°C, the proportion of measurements within this range was calculated (14,15).

There are a number of limitations to this study. A single site for body temperature measurement was compared to the esophageal temperature measurement site, so further investigation into the accuracy of alternative sites is warranted in sheep. These alternative sites include the rectum and tympanic membrane (15). Nasopharyngeal temperature measurements were more accurate than rectal temperature measurements compared with esophageal temperature measurements in anesthetized pigs, but extrapolating across species should be done with caution (14). Another limitation of this study is that the temperature was maintained within a small range during anesthesia. Investigating the influence of high or low body temperature on the correlation between temperature measurements at different sites would also be prudent. The temperature of the fresh gas passed through the breathing system may also affect the accuracy of the nasopharyngeal temperature measurements. The temperature of the fresh gas was not measured but was likely to be close to, or lower than, the ambient temperature (20.5°C to 21.5°C). The temperature of the fresh gas was certainly lower than body temperature but whether a colder fresh gas supply would have influenced the nasopharyngeal temperature measurements is unknown. The fresh gas flow was held for 5 min prior to data collection but a longer period of time at the new fresh gas flow may have been appropriate. Finally the amount of wool growth on sheep may influence the core to peripheral temperature gradient in this species. The insulation provided by wool may stabilize the temperature of sheep to some degree so further investigations into the influence of the thickness of the wool at the time of anesthesia would be of interest.

Based on the low bias, high correlation coefficient, low absolute error, and high percentage of nasopharyngeal temperature measurements that were within 0.5°C of the esophageal measurements, nasopharyngeal temperature measurements were an accurate reflection of the esophageal temperature in anesthetized sheep in this study. The fresh gas flow through the breathing system did not influence the accuracy of the measurements up to 6 L/min of oxygen. Further work is required to elucidate the thermoregulatory capability of sheep at different stages of wool growth during anesthesia and whether other non-invasive sites are appropriate for estimation of core body temperature in this species.

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Cleaning with a wet sterile gauze significantly reduces contamination of sutures, instruments, and surgical gloves in an *ex-vivo* pelvic flexure enterotomy model in horses

Gessica Giusto, Clara Tramuta, Vittorio Caramello, Francesco Comino, Patrizia Nebbia, Patrizia Robino, Ellen Singer, Elena Grego, Marco Gandini

Abstract

The objective of this study was to investigate whether cleaning surgical materials used to close pelvic flexure enterotomies with a wet sterile gauze will reduce contamination and whether the use of a full thickness appositional suture pattern (F) or a partial thickness inverting (or Cushing) suture pattern (C) would make a difference in the level of contamination. Large colon specimens were assigned to group F or C and divided into subgroups N and G. In group G, a wet sterile gauze was passed over the suture material, another over the instruments, and another over the gloves. In group N, no treatment was applied. The bacterial concentration was measured by optical density (OD) at 24 h. The OD of subgroup CG was lower than that of subgroup CN ($P = 0.019$). The OD of subgroup FG was lower than that of subgroup FN ($P = 0.02$). The OD of subgroups CG, CN, FG, and FN was lower than that of the negative control ($P < 0.003$, $P < 0.001$, $P < 0.001$, and $P < 0.00$). The use of a sterile wet gauze significantly reduced contamination of suture materials. A partial thickness inverting suture pattern did not produce less contamination than a full thickness appositional suture pattern.

Résumé

L'objectif de la présente étude était d'examiner si le nettoyage du matériel chirurgical utilisé pour fermer les entérotomies de la courbure pelvienne avec une gaze stérile mouillée réduisait la contamination et si l'utilisation d'un patron de suture d'apposition de la pleine épaisseur (F) ou d'un patron de suture inversé d'une épaisseur partielle (ou Cushing) (C) faisait une différence dans le degré de contamination. Des spécimens du gros côlon ont été assignés au groupe F ou C dans les sous-groupes N et G. Dans le groupe G, une gaze stérile mouillée a été passée par-dessus le matériel de suture, une autre par-dessus les instruments, et une autre par-dessus les gants. Dans le groupe N, aucun traitement ne fut effectué. Les concentrations bactériennes ont été mesurées par densité optique (DO) à 24 h. La DO du sous-groupe CG était inférieure à celle du sous-groupe CN (P = 0,019)*. La DO du sous-groupe FG était inférieure à celle du sous-groupe FN* (P = 0,02)*. Les DO des sous-groupes CG, CN, FG, et FN étaient inférieures à celles des témoins négatifs* (P < 0,003, P < 0,001, P < 0,001, *et* P < 0,00)*. L'utilisation d'une gaze stérile mouillée a réduit de manière significative la contamination de matériel de suture. Un patron de suture inversé avec épaisseur partielle n'a pas entrainé moins de contamination qu'un patron de suture par apposition avec pleine épaisseur.*

(Traduit par Docteur Serge Messier)

Reducing surgical complications is one of the primary aims of surgery. Surgical site infection (SSI) is one of the most common complications encountered in surgery (1). New surgical materials have been created, such as antibiotic-coated suture threads, and surgical procedures have been improved to reduce the risk of surgical site infections and eventually reduce the use of post-operative systemic prophylactic antibiotics (1–4).

Surgical sutures can act as foreign bodies and as a potential nidus for infection, thereby preventing wound healing (5,6). Bacterial adherence to suture materials plays an important role in the development of suture infection (6,7). Thus, in highly contaminated surgical procedures, such as pelvic flexure enterotomy, infections could occur throughout the 2-layer enterotomy closure due to bacterial contamination of the suture. Previous research has shown that monofilament sutures are superior to braided sutures in contaminated wounds because bacteria adhere more tightly to braided sutures, which leads to more infections (6,8). Furthermore, recent studies on incisional infections in humans have established that biofilms occur on suture materials and are responsible for postoperative infections (9). Microorganisms that grow in a biofilm are highly resistant to

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The authors declare that they have no conflict of interest in the publishing of this article.

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antibiotic therapy and can be a serious challenge to eradicate (10). It is therefore important to minimize or prevent contamination at an enterotomy site.

The outcome of equine colic surgery can be influenced by different factors, many of which are beyond the surgeon's control. As treatment modalities, such as preoperative, surgical, and postoperative treatments, are likely to influence outcome, the study of these factors could improve the final result and perhaps increase understanding of some surgical complications. Many aspects of the treatment of surgical colics appear to be influenced by the individual surgeon's preferences, which may benefit from scientific research to identify the best practice to apply (11). One of these could be the pelvic flexure enterotomy (PFE).

Enterotomy is a routine procedure in abdominal surgery in both humans and animals, with enterotomy of the large colon pelvic flexure being particularly common in horses. Although the risk of surgical complications is low, the procedure may cause contamination and inflammation that could lead to postoperative complications, including peritonitis and adhesions (12), either of which can prove fatal to horses. The use of sterile materials and aseptic procedures is therefore mandatory in order to prevent surgical site infections (13). A 2014 survey of current methods used in pelvic flexure enterotomy closure by the European College of Veterinary Surgeons (ECVS) and the American College of Veterinary Surgeons (ACVS) revealed that 58.3% of surgeons adopt measures to reduce contamination during enterotomy closure (14). The most common approach was to close the second layer of the enterotomy with a new suture strand, which is associated with a simultaneous change in surgical gloves and instruments (14). Although some studies reported the use of 2 suture strands to close enterotomies (15), there is no evidence that this technique significantly reduces contamination.

Our main hypothesis in this study was that cleaning the sutures, gloves, and instruments used to close the first layer of a pelvic flexure enterotomy with a wet sterile gauze would significantly reduce bacterial contamination. Our second hypothesis was that a full thickness first layer suture pattern would have the same affect on the level of contamination as an inverting suture pattern. We compared bacterial contamination of surgical materials before and after cleaning with a wet sterile gauze in an *ex-vivo* pelvic flexure enterotomy model in horses.

Samples from the left colon, including the pelvic flexure and approximately 100 cm of ventral colon and 100 cm of dorsal colon, were harvested from 36 horses at a local abattoir immediately after slaughter. Pelvic flexures were placed individually in sterile plastic bags and immersed in sterile 0.9% saline solution at room temperature. The neck of the bag was closed with a plastic band. The pelvic flexure was then cut free from the rest of the large colon. All the tests were conducted within 2 h after death. Samples were randomly assigned (www.random.org) to 2 groups: full thickness (F) and Cushing (C), with 18 samples per group. To mimic clinical settings, each pelvic flexure was placed over a sterile drape on a colon tray. An 8-cm-long incision was made on the antimesenteric site and the luminal content was emptied using a water hose fed into the lumen of the colon. The enterotomy site was continuously lavaged during the procedure with sterile fluids (5-L Ringer's Lactate Solution; SALF SpA, Bergamo, Italy) flushed on the bowel from a standardized height of 30 cm and at a constant rate of 1 L/min. In group F, after the left colon was completely empty, the enterotomy site was closed with a 1-layer, full thickness, simple, continuous pattern suture, whereas in group C, it was closed with a Cushing pattern. All the suture lines were started and ended with a surgeon's knot. These sutures represented the first of a 2-layer suture pattern, which is usually recommended (15). The same experienced surgeon (MG) closed all enterotomy sites using a 2-0 monofilament suture material (Biosyn 2-0; Covidien, Milano, Italy). A new set of sterile instruments and sterile gloves were used to carry out the surgical procedure for each specimen.

Groups F and C were divided into 2 subgroups: not treated (N) and gauze wipe (G), each consisting of 9 samples. In subgroup N, the first 15 cm of suture material and the needle were collected immediately after completion of the suture line. A sterile swab, moistened by immersing in sterile water for 2 s, was passed over the surgical gloves at the level of the 2 distal phalanges of each finger in a proximodistal direction once, in the center of the palmar surface of the finger. Another sterile swab, moistened as before, was passed over the jaws of the needle holder and the tip of the forceps. Each swab and the suture material were placed in a sterile test tube containing a nutrient broth (Oxoid, Milano, Italy) for growing bacteria cells.

In subgroup G, after completion of the suture line, sterile gauze soaked with sterile saline was passed twice over the suture material before culture swabs were collected. Two other sterile, soaked gauzes were used to clean the instruments and the gloves, respectively. Sterile culture swabs were then collected in the same way as for group N. A negative control group was also prepared with 18 samples as for the other groups. Sterile swabs, moistened as reported, were passed over the sterile gloves and instruments before being touched by the surgeon and a new strand of suture material and needle were collected before starting the procedure. As done previously, each swab was placed in a sterile test tube with a nutrient broth.

Collected suture strand and swabs were incubated in nutrient broth at 37°C for 24 h. The bacterial concentration for each sample was then measured by obtaining the optical density (OD) of the broth culture and comparing it to a non-inoculated broth culture (blank/control). Optical density (OD) measurements were taken at 600 nm using the Ultraspec 2000 Spectrophotometer (Pharmacia Biotech, Piscataway, New Jersey, USA).

Statistical power and sample size were calculated using a prefilled Microsoft Excel sheet provided by our statistical department (Microsoft, Redmond, Washington, USA) with the calculated number being 9 per each subgroup. Normal distribution of the data was evaluated with the Shapiro-Wilk test. As this showed that the data were not normally distributed, the Kruskal-Wallis test was used to compare differences in optical density between subgroups for surgical materials. Results were reported as the median (range) and P-values < 0.05 were considered significant. Statistical analysis was done using commercial software (Prism 6.01; GraphPad Software, La Jolla, California, USA).

Results are summarized in Table I. The median OD (min-max) of subgroup CG was 0.054 (0.00 to 0.17) and was significantly lower than subgroup CN, which was 0.1 (0.008 to 0.36; $P = 0.019$). Additionally, the OD of subgroup FG was 0.049 (0.007 to 0.30) and

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Table I. Optical density (OD) results of different groups

	CG	CN	FG	FN	Negative
OD	0.054 (0.00 to 0.17)[a]	0.1 (0.008 to 0.36)	0.049 (0.007 to 0.30)[b]	0.123 (0.015 to 0.27)	0.007 (0.003 to 0.03)[c]

[a] Median OD of CG is significantly lower than CN.
[b] Median OD of FG is significantly lower than FN.
[c] Median OD of negative group was significantly lower than all subgroups.

it was significantly lower than subgroup FN, which was 0.123 (0.015 to 0.27; $P = 0.02$). The difference between subgroups FN and CN and subgroups FG and CG was not significant ($P > 0.999$). Furthermore, the differences between subgroup CG, CN, FG, and FN and the negative control, which was 0.007 (0.003 to 0.03), were significant ($P < 0.003$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively).

Our results demonstrate that after completion of the first layer of pelvic flexure enterotomy closure in horses, wiping with wet sterile gauze ensures a more significant reduction in contamination of suture strand, surgical gloves, and instruments than not wiping. The optical density was similar with a partial thickness inverting (C) or a full thickness appositional (F) pattern of suture. Furthermore, our results demonstrate that a partial thickness inverting suture pattern does not produce significantly less bacterial contamination than a full thickness appositional suture pattern.

It is difficult, if not impossible, to maintain complete asepsis when carrying out a pelvic flexure enterotomy as intraoperative emptying of the colon using a water hose ensures that some ingesta comes in contact with the bowel serosa near the enterotomy site even with aggressive lavage with sterile fluids. Contact between the suture material, gloves, and surgical instruments and the contaminated bowel cannot be avoided when the enterotomy site is closed, which leads to bacterial contamination of the suture material. Therefore, changing the suture material is not sufficient to decrease contamination, as both the instruments and gloves would also have been in contact with the bacteria from the colon (16). The surgeon should assume that the new suture material, instruments, and gloves will likely become contaminated shortly after they come in contact with the area around the enterotomy site. Thus, simply cleaning the suture material, instruments, and gloves with a wet sterile gauze could be effective at reducing the contamination.

An inverting suture pattern does not provide any advantage in terms of contamination of suture material and surgical instruments based on the similar OD values found. The failure to show a difference may result from contact between the surgical material and the highly contaminated edges of the enterotomy site when an inverting pattern is used. An inverting suture pattern may even be detrimental, due to poor bleeding control, poor healing, and excessive inversion of the tissue (15). Hemorrhage control is a crucial factor in the choice of suture pattern, and the full-thickness layer is designed to control bleeding by grasping the mucosa within each needle bite (15).

Although the contamination of suture material and instruments has been reported previously (16,17), only 1 study has evaluated methods to reduce contamination during surgical operations without the use of sterilization or changing suture materials but rather by using isopropyl alcohol (18). The suture material may potentiate the infection at the enterotomy site due to bacterial adherence (17), thus increasing the risk of infection (5,8,17). Inflammation due to suture material increases susceptibility to infection and may cause bacterial biofilm to form, which is difficult to treat and complicates the outcomes of surgical procedures (6,18). This study did not establish that biofilm formation would be decreased or prevented. The fact that the median optical density of the group using gauze (G) was 7 times that of the median optical density of the negative control (N) suggests that a substantial number of bacteria is still present on the suture, instruments, and gloves. It could be speculated that wiping with a gauze may be enough to diminish the biofilm formation, but this cannot be proved with this study. Nevertheless, a surgeon who carries out an enterotomy should always follow good surgical practice to minimize contamination as much as possible. During closure of an equine pelvic flexure enterotomy, if the first layer of the closure is contaminated, then minimizing contamination for the second layer of closure should effectively reduce inflammation and procedural complications.

The present study is limited because it was conducted in an *ex-vivo* setting, which did not allow us to evaluate all the factors that may contribute to potential complications, including postoperative adhesion formation, in order to assess the effectiveness of our method. However, the conditions of each enterotomy were similar and the health status of the animal or the degree of ischemia of the pelvic flexure were not factors in determining bacterial levels. In this study, although we did not assess the type of bacteria present after suture and swab incubation, we evaluated a method to clear adherent bacteria, independently of their species. Although the study was carried out using the same instruments and conditions as in a surgical theater, it is important to note that different *in-vivo* factors may contribute to the healing of highly contaminated wounds.

Although we didn't compare monofilament and multifilament suture materials, we chose a monofilament suture material that withstands contamination better than multifilament sutures (8,17,19–20). Different results may be obtained with different suture materials. The type of sampling used could be seen as another limitation of this study. Results may have been different if the tips of the instruments and the fingers of the gloves were immersed directly in the nutrient broth and incubated instead of using moistened swabs. We decided to consider the contamination of gloves, instruments, and suture material as a total and not differentiate among the contaminations. This situation could be seen as a limitation, but it was decided to assume that, if only one of these materials still presented a contamination risk after the procedure of wiping, all of the materials would immediately become contaminated again, thereby canceling the effect of the sterile gauze.

In conclusion, we can state that a partial thickness inverting suture pattern in the first layer of a pelvic flexure enterotomy does not reduce contamination of suture material, instruments, and gloves compared with a full-thickness pattern. Cleaning the suture

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material, instruments, and gloves with a wet sterile gauze significantly reduces contamination at the PFE site. Based on the findings of this study, we can speculate that application of this method in a clinical setting should reduce bacterial contamination of pelvic flexure enterotomies in horses.

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Characterization and therapeutic application of canine adipose mesenchymal stem cells to treat elbow osteoarthritis

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Abstract

Visceral adipose tissue (AT) obtained from surgical waste during routine ovariectomies was used as a source for isolating canine mesenchymal stem cells (MSCs). As determined by cytofluorimetry, passage 2 cells expressed MSC markers CD44 and CD90 and were negative for lineage-specific markers CD34 and CD45. The cells differentiated toward osteogenic, adipogenic, and chondrogenic directions. With therapeutic aims, 30 dogs (39 joints) suffering from elbow dysplasia (ED) and osteoarthritis (OA) were intra-articularly transplanted with allogeneic MSCs suspended in 0.5% hyaluronic acid (HA). A highly significant improvement was achieved without any medication as demonstrated by the degree of lameness during the follow-up period of 1 y. Control arthroscopy of 1 transplanted dog indicated that the cartilage had regenerated. Histological analysis of the cartilage biopsy confirmed that the regenerated cartilage was of hyaline type. These results demonstrate that transplantation of allogeneic adipose tissue-derived mesenchymal stem cells (AT-MSCs) is a novel, noninvasive, and highly effective therapeutic tool in treating canine elbow dysplasia.

Résumé

Du tissu adipeux viscéral (TA) obtenu de résidus chirurgicaux lors d'ovariectomies de routine a été utilisé comme source pour isoler des cellules souches mésenchymateuses canines (CSMs). Tel que déterminé par cytofluorométrie, les cellules du 2e passage exprimaient les marqueurs de CSM CD44 et CD90, et étaient négatives pour les marqueurs spécifiques de lignée CD34 et CD45. Les cellules se sont différenciées dans des directions ostéogéniques, adipogéniques, et chondrogéniques. À des fins thérapeutiques, 30 chiens (39 articulations) souffrant de dysplasie du coude (DC) et d'ostéoarthrite (OA) ont reçu une transplantation intra articulaire de CSMs allogéniques en suspension dans 0,5 % d'acide hyaluronique (AH). Une amélioration hautement significative a été obtenue sans aucune médication tel que démontré par le degré de boiterie durant la période de suivi de 1 an. Une arthroscopie de contrôle de un des chiens ayant reçu une transplantation montrait que le cartilage s'était régénéré. L'analyse histologique de la biopsie du cartilage a confirmé que le cartilage régénéré était de type hyalin. Ces résultats démontrent que la transplantation de cellules souches mésenchymateuses dérivées de tissu adipeux allogène est un outil thérapeutique novateur, non-invasif, et très efficace pour traiter la dysplasie du coude chez le chien.

(Traduit par Docteur Serge Messier)

Osteoarthritis (OA) is a progressive degenerative disease of the joint; its symptoms include synovial inflammation, cartilage loss, torn ligaments, osteophyte formation, and joint space narrowing (1). All of these lead to increased pain sensation, stiffness of the joint, lameness, and often a severely decreased quality of life. While the exact cause of OA is not known, it is associated with both environmental factors, such as obesity, age, and damage of the joint, as well as genetic factors, including dysplasia (2).

Regenerative medicine provides novel tools to repair damaged cartilage in joints as demonstrated by a plethora of research articles and clinical trials focusing on the role of chondrocytes and mesenchymal stem cells (MSCs) in treating OA (3). As MSCs are multipotent stromal cells that lack specific surface markers, the International Society for Cellular Therapy has declared that MSCs must meet 3 criteria, they should: i) grow as adherent cells; ii) express CD44, CD90, and CD105, while lacking endothelial and hematopoietic markers CD45, CD34, CD14 or CD11b, CD79α or CD19, as well as HLA-DR; and iii) be able to differentiate into chondrocytes, adipocytes, and osteocytes (4).

Most studies have analyzed the function of bone marrow MSCs (BM-MSCs) and adipose tissue-derived MSCs (AT-MSCs) (5). While AT-MSCs are similar to BM-MSCs in terms of their usefulness in regenerative medicine, they have an important advantage over BM-MSCs, namely, that they can be isolated in larger numbers using a minimally invasive procedure (5). As AT-MSCs are non-immunogenic and have a potent immunosuppressive activity, they can be used safely in allogeneic or even xenogeneic transplantation in OA and other models without the need for immunosuppressive

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medications (6,7). To enhance their effect, improve MSC survival, and ensure the prolonged presence of MSCs in a harsh microenvironment, MSCs can be combined with traditional treatments such as injecting hyaluronic acid (HA) (8,9).

The objective of this study was to evaluate the long-term effects of administering MSCs dispersed in an HA solution on regeneration of osteoarthritic elbow joints in dogs. Allogeneic AT-MSCs were isolated from surgical waste obtained from the spaying of female mixed-breed dogs and were characterized by marker analysis and differentiation. The cells were suspended in HA and injected into joints of dogs suffering from elbow dysplasia with OA. The treatment efficacy was assessed using questionnaires completed by the dog owners based on changes in the dogs' lameness. Transplantation of MSCs resulted in highly significant long-term improvement (over a 1-year period) in the condition of the involved animals.

This study was approved by the National Scientific Ethical Committee of the National Food Chain Safety Office. All the dog owners signed an informed consent authorizing treatment and were informed of the possible risks of joint injections and potential complications of the procedure.

Visceral adipose tissue (AT) was collected by a veterinarian from 13 healthy, small, mixed-breed dogs that underwent all routine vaccinations and were regularly surveyed by veterinarians. Adipose tissue was digested with 0.1% collagenase (Sigma Aldrich, St. Louis, Missouri, USA) in Hank's buffer for 30 min at 37°C. The resulting stromal vascular fraction (SVF) was plated at $10^4/cm^2$ in DMEM/F12 containing 10% fetal calf serum (FCS), 2 mM glutamine, and 50 U/mL penicillin and streptomycin (complete medium) (all from Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cultures were grown up to 80% confluency, diluted twice to reach stage passage 2 (P2), and stored in liquid nitrogen until further usage.

Passage 2 (P2) cells from 8 donors were analyzed for surface markers by cytofluorimetry (FACSCalibur; BD Biosciences, San José, California, USA) using the following antibodies: fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse/human CD44 (BioLegend, San Diego, California, USA); Phycoerythrin (PE)-conjugated rat anti-canine CD90 (eBioscience, San Diego, California, USA); FITC-conjugated rat anti-canine CD45 (eBioscience); and FITC-conjugated mouse anti-canine CD34 (R&D Systems, Minneapolis, Minnesota, USA). The data were evaluated using CellQuest software (BD Biosciences).

The differentiation capacity of P2 cells was tested as follows. The cells were fixed in 8% formaldehyde in phosphate-buffered saline (PBS) for 15 min after culturing cells in osteogenic differentiation medium (10 mM β-glycerophosphate, 50 μg/mL L-ascorbic acid 2-phosphate, and 10 nM dexamethasone, all from Sigma Aldrich) for 21 d (10) or adipogenic differentiation medium (15% rabbit serum, 2 mM glutamine, and 50 U/mL penicillin and streptomycin containing DMEM/F12) for 4 d (11). The cultures were then stained with 1% Alizarin Red S for 45 min or AdipoRed (Lonza, Basel, Switzerland) for 15 min and analyzed with an Olympus CKX41 microscope at 200× magnification or a fluorescent Olympus microscope (Olympus IX81) at 100× magnification, respectively. Photos were taken with an Olympus C-5060 digital camera from 5 non-overlapping regions for osteogenic and adipogenic differentiation.

Chondrogenic differentiation was induced according to Bosnakovski et al (12) in 3-dimensional pellet culture without any growth factor for 21 d. Fifteen chondrogenic pellets were collected and digested with 0.3% collagenase (Sigma Aldrich) in Hank's buffer containing 2 U/μL RNase inhibitor (RNasin Promega, Madison, Wisconsin, USA) for 30 min at 37°C in a water bath. Total ribonucleic acid (RNA) was isolated from the pellet cultures and from non-induced cells as control using a Perfect Pure RNA Kit (5Prime, Gaithersburg, Maryland, USA) according to the manufacturer's instructions. A RevertAid H Minus First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to obtain complementary deoxyribonucleic acid (cDNA) from 94 ng of total RNA/reaction. Quantitative real-time polymerase chain reaction (qPCR) was carried out using an AccuPower 2X Greenstar qPCR Master Mix (Bioneer, Daejeon, Republic of Korea) in a RotoGene3000 instrument (Qiagen, Hilden, Germany).

Relative quantification of gene expression was done by comparing threshold values. All results were normalized to *Gapdh* (*Gapdh:* forward: TGGCAAAGTGGATATTGTCG reverse: AGATG GACTTCCCGTTGATG) and qPCR primers (*Sox9:* forward: ACGAC TACACTGACCACCAGAAC reverse: GTAGGTGAAGGTGGAGTAG AGGC, *Aggrecan:* forward: TTGCACTCAGGAGAGGAGAC reverse: CCACGCAGGTGGCTCCATTC) were used as in Lee et al (13) and Zang et al (14), with slight modifications.

Osteogenic, adipogenic, and chondrogenic differentiation assays were carried out on MSC cultures derived from 3 donors for osteogenic, 1 donor for adipogenic and 1 donor for chondogenic differentiation.

Preparation of AT-MSCs for therapy

Passage 2 AT-MSCs from 2 different adipose tissue donors were thawed, cultured for 3 d, mixed, and suspended in 0.5% sodium hyaluronate (TRB Chemedica International S.A. Geneva, Switzerland). Adipose tissue-derived MSCs ($12 \times 10^6 \pm 3.2 \times 10^6$ cells/injection) were transported to the veterinarian clinics in syringes. In total, 6 different MSC cultures derived from 6 donors were used for transplantation.

Patient selection

Thirty-nine elbow joints from 30 dogs were included in the study. The inclusion criteria were recurrent lameness and pain attributed to OA after conventional treatment of dogs suffering from elbow dysplasia and cartilage degeneration (nonsteroidal anti-inflammatory drugs, intra-articular injection of hyaluronic acid, arthroscopy, or traditional surgery). Five dogs with osteoarthritic elbows were excluded as they were receiving pain relief medication for additional hip dysplasia or were undergoing analgesic treatment for spinal disc herniation. During this study, 6 dogs with elbow dysplasia died due to diseases unrelated to transplantation. Anti-inflammatory drugs (carprofen or meloxicam) were administered 10 d before transplantation.

The health status of the treated dogs was evaluated by the owners using a questionnaire modified from Black et al (15), as well as a survey by the veterinarians. The veterinarians taking part in the survey also collected data regularly from the owners and the results at 6, 9, and 12 mo were used to assess the effectiveness of stem cell treatment.

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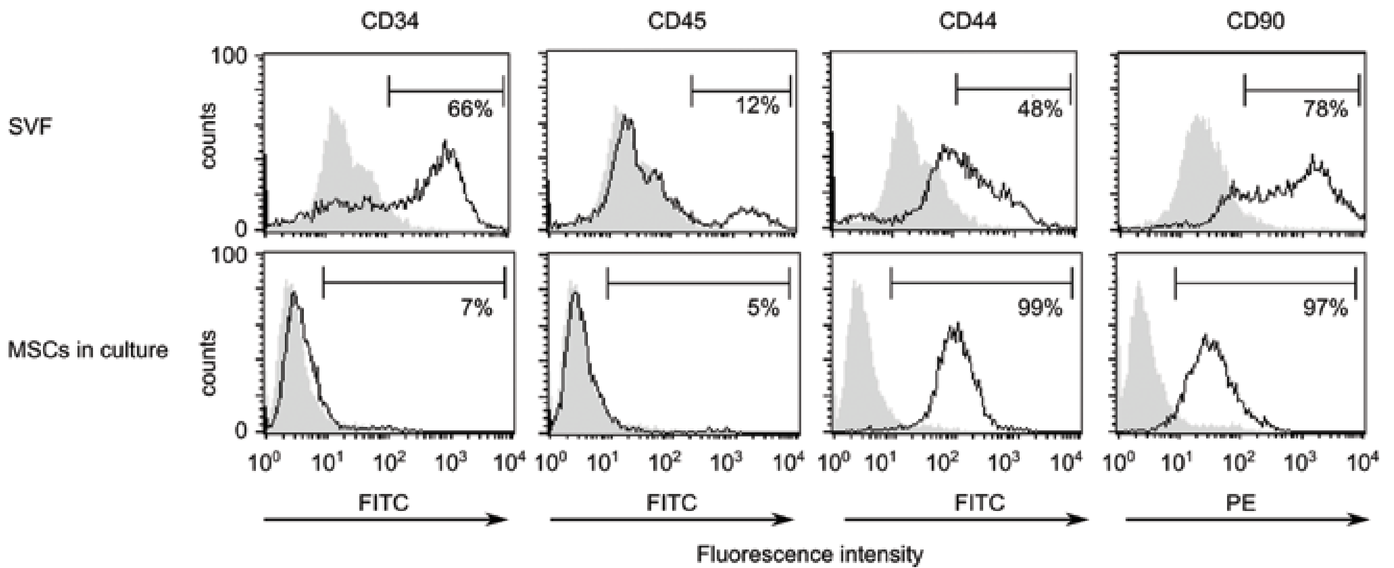

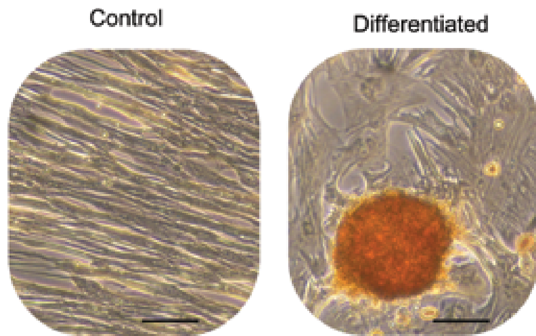

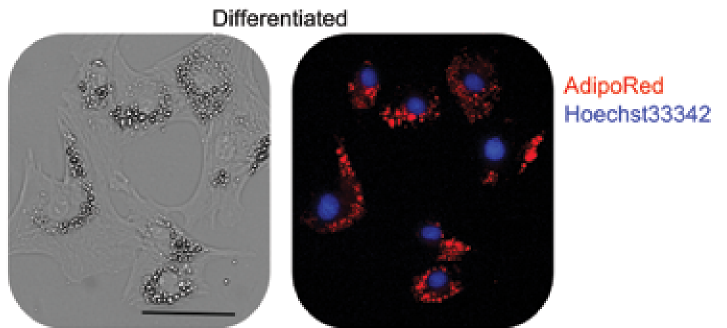

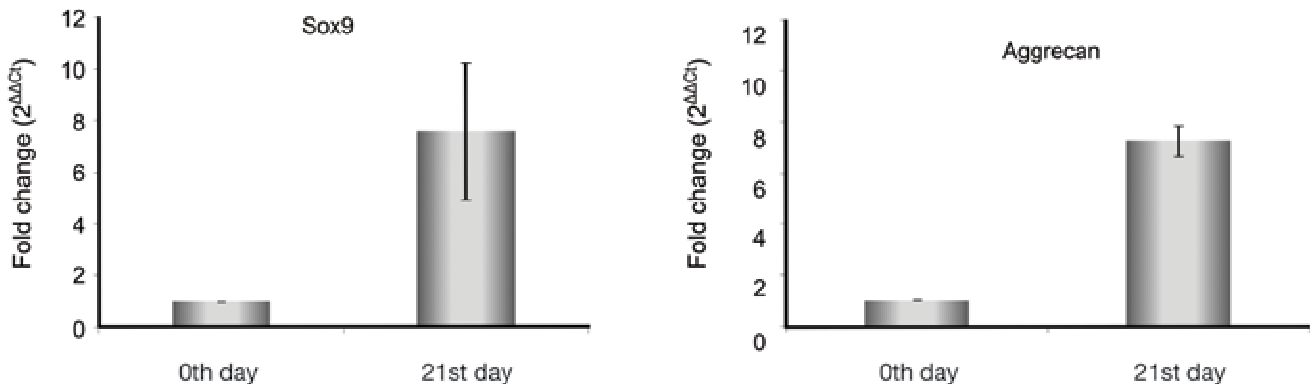

Figure 1. Characterization of adipose tissue-derived mesenchymal stem cells (AT-MSCs). A — The presence of $CD34^+$, $CD45^+$, $CD44^+$, and $CD90^+$ cells was analyzed in isolated stromal vascular fraction (SVF) (top row) and cultured AT-MSC (bottom row) with flow cytometer. Grey histograms represent control samples without any antibody and black-lined histograms show fluorochrome-conjugated, antibody-stained samples. Percentages indicate the proportion of positive cells to the analyzed surface marker in the cell population. B — Osteogenic differentiation. Extracellular calcium was stained with Alizarin Red S solution. Representative figure shows matrix mineralization in an induced passage 2 cell culture. Original magnification: 200×, scale bar = 500 μm. C — Adipogenic differentiation. Accumulated lipid droplets were stained with AdipoRed (red) and nuclei were dyed with Hoechst 33342 (blue). Original magnification: 100×, scale bar 100 μm. D — Chondrogenic differentiation. Chondrogenic induction was carried out in micromass culture and quantitative polymerase chain reaction (qPCR) was conducted for mRNA expression of *Sox9* and *aggrecan.* Cells on day 0 of micromass induction served as control. Results are expressed as mean ± standard deviation.

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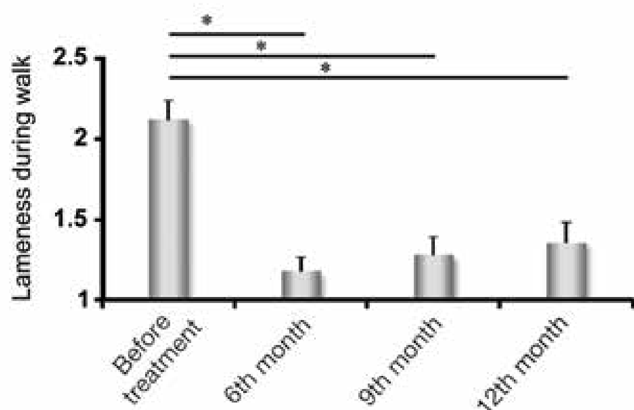

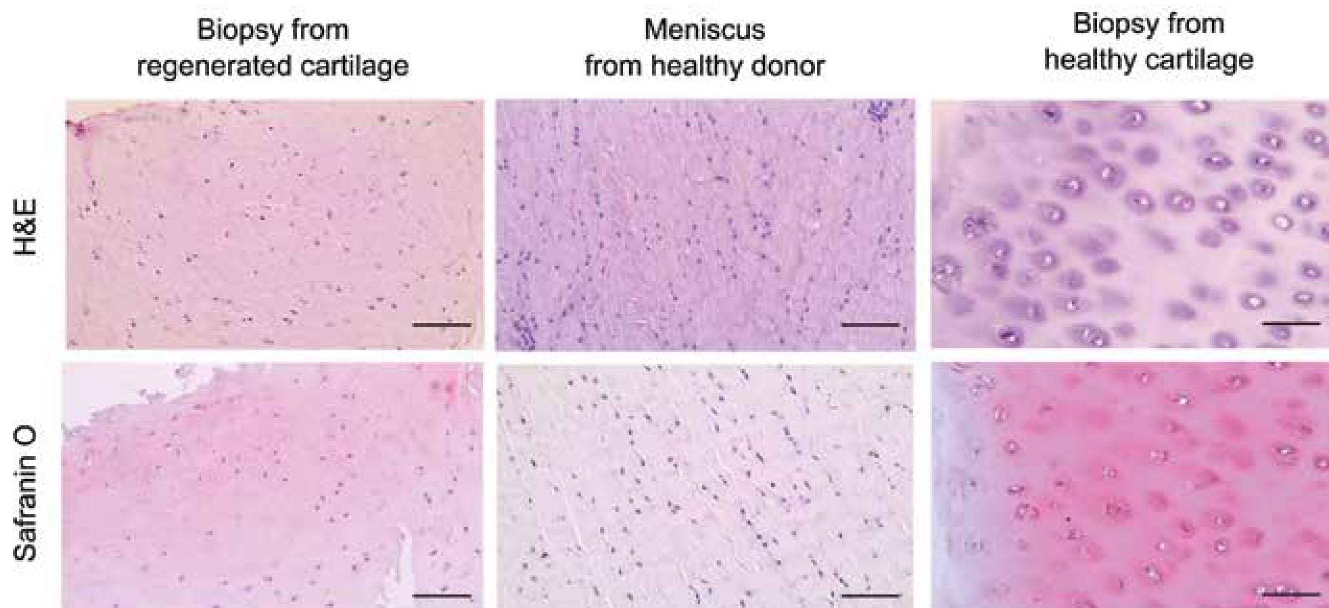

Figure 2. Therapeutic effect of adipose tissue-derived mesenchymal stem cells (AT-MSCs). A — Lameness was rated by the owners using a questionnaire modified from Black et al (15). Results were substantiated by the nonparametric Wilcoxon test. $^*P < 0.001$, N = 39. B — Images were excised from videos taken during arthroscopic surgery 3 wk before MSC injection (left image) and control arthroscopy 1 y after transplantation (right image). Images represent the same area of the joint. C — Images of tissue samples at 200× magnification, stained with hematoxylin and eosin (H&E, upper row) and Safranin O (lower row), obtained from the regenerated cartilage (left column), a healthy meniscus cartilage (middle), and healthy cartilage (right column). Scale bar = 50 μm.

Arthroscopic record samples were taken from 1 dog before cell injection and 12 mo after transplantation when the biopsy sample was also taken. As the dog's diagnosis was fissured medial coronoid process, a subtotal coronoidectomy was carried out and the torn part was removed. Images were copied from the video, which referred to the same site of the ulna. As the original video was taken at low resolution, we were not able to present better quality images. The biopsy specimen was taken from the regenerated area of the cartilage of 1 dog during arthroscopic survey. After fixation in 10% neutral buffered formalin for 24 h at room temperature, tissue samples were dehydrated in a series of ethanol and xylene baths and embedded in paraffin wax. Sections (3 to 4 μm) were stained with hematoxylin and eosin or Safranin O. The slides were analyzed using an Olympus BX53 light microscope and images were taken with an Olympus SC100 digital camera using the Olympus cellSense software. Data were calculated as mean ± standard error of mean (SEM) and groups were compared using the nonparametric Wilcoxon test.

Cell surface markers of the initial SVF and cultured MSCs were analyzed. The stromal vascular fraction (SVF) contained cells with lineage-specific markers (66% $CD34^+$ hematopoietic progenitor and

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endothelial cells and 12% CD45$^+$ leucocytes) and cells with MSC markers (48% CD44 and 78% CD90). After culturing, the contaminating cell lineages disappeared and the resulting homogenous CD44$^+$ and CD90$^+$ cell population was referred to as AT-MSCs (Figure 1A). Passage 2 AT-MSCs were analyzed for differentiation capacity. The results showed that AT-MSCs differentiated into osteoblasts (Figure 1B) and adipocytes (Figure 1C), as verified by Alizarin Red for extracellular calcium and AdipoRed for lipid droplet staining. Elevation of *aggrecan* and *Sox9* messenger RNA (mRNA) expression was detected when chondrocyte differentiation was induced (Figure 1D).

Thirty dogs (39 joints) suffering from elbow dysplasia resulting in OA and cartilage degeneration were treated with AT-MSCs and followed for 1 y by veterinarians and the owners. Based on the owners' evaluations, AT-MSCs had significant effect (31 cases out of 39) on the dogs' lameness during walking (Figure 2A) without any further medication for at least a 1-year period. Three further joints from the 39 showed stable improvement in lameness, with only occasional application of painkillers required.

Comparison of arthroscopic images taken before and after transplantation showed regenerated cartilage islets in the MSC-treated joint (Figure 2B). Analysis of hematoxylin-eosin stained slides revealed that the repaired tissue was hyaline-like cartilage with fibrous elements (upper left, Figure 2C) compared to hyaline cartilage from a healthy joint (upper right, Figure 2C) and it was morphologically very different than fibrocartilage obtained from a healthy dog, which is typically found in the meniscus (upper middle, Figure 2C). The regenerated cartilage and healthy hyaline cartilage had lower cell density and less fibrous elements than fibrocartilage (Figure 2C). Moreover, Safranin O staining indicated higher glycosaminoglycan (GAG) content in the regenerated cartilage (lower left, Figure 2C) and healthy hyaline cartilage (lower right, Figure 2C) compared to fibrocartilage (lower middle, Figure 2C).

Damage from osteoarthritis and cartilage does not heal spontaneously due to poor vascularization and high density synovial fluid. There is therefore a great need for novel therapies to enhance formation of proper cartilage as well as to inhibit and cure OA. This is especially the case for elbow dysplasia, for which there are far fewer surgical solutions than for hip dysplasia. Recent developments include the local transplantation of MSCs into the articular cavity. This study shows that visceral adipose tissue is an excellent source of canine MSCs. After isolation, MSCs showed the expected characteristics, including the expression of cell surface markers, CD90 and CD44. Moreover, they were free of the lineage-specific markers CD45 and CD34 and retained their differentiation capability, since they differentiated toward osteogenic, adipogenic, and chondrogenic directions.

Intra-articular injection of AT-MSCs resulted in highly significant and sustained improvement in the condition of the patients. Lameness was ameliorated after 3 mo of treatment (data not shown) without any further medication and this improvement persisted at least during the 1-year survey. To our knowledge, this is the longest survey carried out with the largest patient population in the literature. It is worth mentioning that several additional dogs suffering from osteoarthritis in joints other than the elbow have also been treated with MSCs with encouraging results. The dogs' conditions improved after MSC transplantation in 3 out of 4 hip cases, 2 out of 2 shoulder cases, 3 out of 3 knee cases, and 3 out of 3 hock joints (data not shown). Side effects were rare, with swelling for several days in 2 of 39 treated joints. Comparison of surgical and control arthroscopy confirmed newly regenerated cartilage after treatment. Although fibrocartilage tissue is formed during spontaneous repair of damaged hyaline cartilage, our results clearly indicate that MSC transplantation induces hyaline-like cartilage formation, which is more adequate for proper joint function. Our results strongly indicate that the significant improvement in lameness is attributable to the formation of long-lasting (sustained), hyaline-like cartilage repair tissue due to AT-MSC transplantation.

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