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Changes in antimicrobial resistance levels among Escherichia coli, Salmonella, and Campylobacter in Ontario broiler chickens between 2003 and 2015

Agnes Agunos, Richard K. Arsenault, Brent P. Avery, Anne E. Deckert, Sheryl P. Gow, Nicol Janecko, David F. Léger, E. Jane Parmley, Richard J. Reid-Smith, Scott A. McEwen

Abstract

Poultry has been identified as a reservoir of foodborne enteric pathogens and antimicrobial resistant bacteria. The objective of this study was to describe and compare antimicrobial resistant isolates from an Ontario broiler chicken farm-level baseline project (2003 to 2004) to the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) Ontario abattoir and retail surveillance data from 2003, and to the most recent (2015) CIPARS Ontario chicken surveillance data in order to assess the impact of an industry-wide policy change in antimicrobial use. Cefiofur resistance (TIO-R) prevalence in Salmonella decreased by 7% on farm between 2003 and 2004 and 2015. During the same timeframe, TIO-R E. coli prevalence decreased significantly by 16%, 11%, and 8% in farm, abattoir, and retail samples, respectively. Gentamicin resistant (GEN-R) E. coli, however, increased by 10% in farm and 15% in retail-derived isolates, and trimethoprim-sulfamethoxazole resistant (TMSm-R) E. coli increased significantly by 20%, 18%, and 5% in farm, abattoir, and retail isolates, respectively. Similarly, ciprofloxacin-resistant (CIP-R) Campylobacter spp. significantly increased in retail isolates by 11% and increased in farm (33%) and abattoir isolates (7%). The decrease in TIO-R Salmonella/E. coli in recent years is consistent with the timing of an industry-led intervention eliminating the preventive use of cefiofur, a third generation cephalosporin and class of antimicrobials deemed critically important to human medicine. The rise in GEN-R and TMSm-R prevalence is indicative of recent shifts in antimicrobial use. Our study highlights the importance of integrated surveillance in detecting emerging trends and determining the efficacy of interventions to improve food safety.

Résumé

La volaille a été identifiée comme étant un réservoir d’agents pathogènes entériques d’origine alimentaire et de bactéries résistantes aux antimicrobiens. L’objectif de la présente étude était de décrire et comparer des isolats résistants aux antimicrobiens provenant d’une ferme ontarienne de poulets à griller obtenus dans le cadre d’un projet de base (2003 à 2004) aux données de surveillance de 2003 du Programme intégré canadien de surveillance de la résistance aux antimicrobiens (PICRA) d’abattoir et de ventes au détail en Ontario, et aux plus récentes données de surveillance (2015) du PICRA Ontario pour la volaille afin d’évaluer l’impact d’un changement à l’ensemble de l’industrie dans l’utilisation des antimicrobiens. La prévalence de la résistance au cefiofur (TIO-R) de Salmonella a diminué de 7 % sur la ferme entre 2003 à 2004 et 2015. Durant ce même intervalle de temps, la prévalence de TIO-R de E. coli diminua de manière significative de 16 %, 11 %, et 8 % dans les échantillons provenant de la ferme, de l’abattoir et de la vente au détail, respectivement. Toutefois, les E. coli résistants à la gentamicine (GEN-R) ont augmenté de 10 % et 15 % dans les échantillons pris à la ferme et de la vente au détail, respectivement. Les E. coli résistants au trimethoprime-sulfaméthoxazole (TMSm-R) ont augmenté de manière significative par 20 %, 18 %, et 5 % dans les isolats de la ferme, de l’abattoir et de la vente au détail, respectivement. Les isolats de Campylobacter spp. résistants au ciprofloxacine (CIP-R) augmentèrent de manière significative dans les échantillons de vente au détail (11 %), ceux de la ferme (33 %) ainsi que ceux de l’abattoir (7 %). La diminution de la TIO-R chez Salmonella/E. coli au cours des dernières années concorde avec une intervention menée par l’industrie d’utiliser l’utilisation en prévention du cefiofur, une céphalosporine de troisième génération qui est une classe d’antimicrobiens considérée d’importance critique en médecine humaine. L’augmentation de la prévalence de GEN-R et de TMSm-R est indicative d’un changement récent dans l’utilisation des antimicrobiens. Notre étude fait ressortir l’importance d’un programme intégré de surveillance pour détecter les tendances émergentes et déterminer l’efficacité des interventions pour améliorer la salubrité alimentaire.

Introduction

Contamination of food with Salmonella and Campylobacter remains a significant food safety issue among Canadians (1,2), and resistant Salmonella and Campylobacter strains pose an additional food safety threat. The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) monitors antimicrobial resistance (AMR) in the food chain in Canada. The flow of products from hatching egg production to retail in Canada is vertically coordinated but not integrated, unlike other countries. The CIPARS has current surveillance activities to detect AMR in the farm, abattoir, and retail stages of the broiler chicken production chain. Of particular concern is the detection of bacterial strains resistant to antimicrobials deemed critically important to human medicine by the World Health Organization...
In the last decade, there has been increasing public awareness about microbiological food safety. This has resulted in on-farm food safety enhancements (6) and other farm-based initiatives. Vaccination against Salmonella is one such strategy that has been used in laying hens to reduce the duration, shedding, and potential for re-infection (7,8). Controlling Salmonella in the breeder sector also reduces the potential for downstream contamination throughout the poultry production chain. In Ontario, serovar-specific control practices are being utilized in breeder flocks. These strategies include termination of flocks found positive for Salmonella enterica enterica serovar Enteritidis and S. Typhimurium DT104, as well as vaccination against S. Enteritidis, S. Typhimurium, S. Heidelberg, and S. Kentucky (9).

Veterinarians in Ontario routinely diagnose bacterial diseases in the field and there is evidence that poultry pathogens have persisted (e.g., avian pathogenic E. coli, Clostridium perfringens) and emerged (e.g., Enterococcus cecorum) (10). Availability of antimicrobials in Canada for therapy of these diseases may become more limited due to AMR development or decreased access. Limited access may result from clinically normal slaughtered chickens at the abattoir (4). In Ontario, abattoir and retail surveillance during the same period also found changes in prevalence of TIO-R E. coli and Salmonella; however, there were no corresponding antimicrobial use (AMU) data available to explain these findings (5).

Although CIPARS has tracked changes in the occurrence of antimicrobial resistance in Salmonella, E. coli, and Campylobacter in chickens at slaughter and chicken meat at retail since 2002 to 2003 (5,16), CIPARS did not have a broiler chicken farm component for comparison to abattoir and retail data until 2013. There was, however, a farm-based broiler chicken pathogen project conducted in Ontario in 2003 to 2004, which had many methodological similarities to the current CIPARS broiler-farm component. The objective of the present study was to describe the farm-level Salmonella serovar distribution and AMR among E. coli, Salmonella, and Campylobacter isolates from samples collected from Ontario broiler flocks as part of a farm-level pathogen baseline project conducted in 2003 to 2004 and compare these data to the CIPARS Ontario abattoir and retail surveillance data from 2003. In the context of industry-wide changes in antimicrobial use policy, a second objective was to compare the 2003 to 2004 farm-level baseline project data to the most recent (2015) CIPARS Ontario farm, abattoir, and retail surveillance data.

Broiler chicken sampling

Ontario broiler farm study (OBFS) farm isolates, 2003 to 2004.

In this paper, the 2003 to 2004 OBFS refers to an Ontario Ministry of Agriculture, Food and Rural Affairs-funded farm-level Salmonella and Campylobacter baseline research project conducted in Ontario broiler flocks. Flocks were selected from a list of broiler chicken producers that volunteered in the OBFS. The study is described in detail elsewhere (17). In brief, various farm environment (e.g., clean litter and surface swabs in bird contact areas) and bird-associated samples (fecal samples, cloacal swabs, and meconium swabs from chick pads) were collected from single flocks on 90 commercial broiler chicken farms in Ontario between May 2003 and May 2004. Equal numbers of farms were sampled from the 9 administrative districts of the Chicken Farmers of Ontario. One flock (defined as a group of birds hatched at the same time, delivered, and reared in the same barn or pen/floors) per farm (a registered establishment in Ontario and may have multiple barns in the premise) was sampled twice. The first sampling occurred on the day a new crop of chicks from the hatchery (chick placement) was delivered and then a second sampling was conducted 1 to 7 d before the flock was sent for slaughter (pre-harvest). Of the 90 flocks initially sampled, pooled fecal and environmental samples from 50 flocks were frozen in 2 mL aliquots and stored at −82°C for possible future analysis.

The CIPARS farm, abattoir, and retail samples. In 2015, the CIPARS farm program collected samples using a similar methodology to that described. Briefly, to ensure representativeness, the following farm inclusion and exclusion criteria were utilized: farms needed to be Safe, Safer, Safest [i.e., a comprehensive food safety and biosecurity program monitored by the Chicken Farmers of Canada (6)] compliant, representative of the hatcheries and feedmills supplying chicks and feeds in Ontario, and representative of the veterinary practice profile of the participating veterinarian. Samples were also collected at placement (environmental and chick pad swabs) and pre-harvest (fecal). In the CIPARS abattoir component, fecal samples were collected from clinically normal slaughtered birds in all 7 federally registered Ontario abattoirs processing commercial broiler chickens. In the CIPARS retail component, fresh chicken meat (legs and/or thighs bone-in with skin-on) were collected from grocery stores/butcher shops from population weighted, randomly selected census divisions in Ontario (18).

Bacterial isolation and antimicrobial susceptibility testing

For the recovery of Salmonella, fecal samples were enriched in buffered peptone water (BPW) (1:10 ratio) and incubated at 35°C for 24 h. A 0.1-mL loopful of BPW broth was inoculated into a modified semi-solid Rappaport Vassiliadis (MSRV) plate and incubated at 42°C for 24 to 72 h. For E. coli, a loopful of BPW broth was inoculated on MacConkey agar and incubated at 35°C for 24 h. Further isolation and characterization of each organism has been previously described (18). Archived Salmonella isolates from the OBFS had been stored at −82°C at the Office Internationale des Epizooties Reference Laboratory for Salmonellosis, Salmonella Typing Laboratory, National...
Microbiology Laboratory (NML) at Guelph [formerly the Laboratory for Foodborne Zoonoses (LFZ)], Public Health Agency of Canada, Guelph, Ontario, and were grown in Mueller-Hinton agar followed by phenotypic susceptibility testing using microbroth dilution methods. Generic *E. coli* isolates were cultured from aliquots of pooled fecal samples and fecal and chick paper pad samples using routine *E. coli* culture methodology (18) at the University of Guelph and transferred to NML at Guelph for antimicrobial susceptibility testing.

*Campylobacter* isolates from the OBFS were originally processed by the Agri-food Laboratory, University of Guelph. Briefly, environmental swab samples were enriched in Rosell’s broth (microaerophilically, temperature was adjusted in a stepwise manner at 30°C, then to 37°C, then at 43°C for a total of 48 to 72 h). The fecal samples were directly plated onto Campy-Charcoal media and were incubated in a microaerophilic environment at 37°C for 72 h, after further characterization, the resulting isolates were stored in *Brucella* broth with 15% glycerol at −82°C (17). In 2015, these broth cultures were transported on dry ice to the NML at St. Hyacinthe (formerly LFZ), PHAC, St. Hyacinthe, Quebec for antimicrobial susceptibility testing. All isolates were also typed by polymerase chain reaction (PCR) according to routine CIPARS protocols (18).

Minimum inhibitory concentrations (MIC) for the 3 bacterial organisms were determined using an automated broth microdilution and the Clinical and Laboratory Standard Institute (CLSI) M7-A8 standards and breakpoints when available (18). The *E. coli* and *Salmonella* isolates were susceptibility tested using routine CLSI protocols and the CMV2AGNF plate (Sensititre; Trek Diagnostic Systems, West Sussex, England) designed by the National Antimicrobial Resistance Monitoring System (NARMS) of the United States. All *Campylobacter* isolates were also susceptibility tested using routine CIPARS protocols and the NARMS CAMPY plates (Sensititre; Trek Diagnostic Systems) (18).

Antimicrobial use

Farm and hatchery level AMU and other farm-level operational and biosecurity information were collected for the CIPARS farm program using questionnaires administered by the participating veterinarian to the producer (18). The AMU information was not available for the OBFS.

Statistical data analysis

Analysis was done using computer software (Stata 13; StataCorp, College Station, Texas, USA). At the isolate level, for each organism, data were dichotomized into susceptible (including intermediate susceptibility) or resistant using CIPARS breakpoints. If no CLSI interpretative criteria were available for a specific antimicrobial/organism combination, breakpoints were based on the distribution of MIC and harmonized with those of the NARMS (18). Resistance prevalence estimates were then adjusted for clustering at the flock level using generalized estimating equations (GEE) with a binary outcome, logit-link function, and exchangeable correlation structure. Null binomial response models were run for each antimicrobial and from each null model, the intercept ($\beta_0$) and 95% confidence intervals (CI) were used to calculate population-averaged prevalence estimates using the formula $[1 + \exp(-\beta_0)]^{-1}$.

**Temporal analysis.** TIO, gentamicin (GEN), trimethoprim-sulfamethoxazole (TMSm), and CIP resistance data from the 2003 to 2004 OBFS, and CIPARS data from 2003 abattoir and 2003 retail components were compared to the 2015 CIPARS data from farm, abattoir, and retail using logistic regression models (asymptotic or exact models depending on prevalence of the outcome variable). Models were developed with year as a categorical independent variable and using $P \leq 0.05$ for significance (i.e., marked by the use of the words “significant” or “significantly” throughout the text). Trends in the percentages of total *Salmonella* of 5 selected *Salmonella* serovars, resistance prevalence and AMU were also analyzed descriptively. Hereafter, significant changes in resistance prevalence are simply referred to as changes in resistance. The percent change in the proportion of multi-class resistance [i.e., resistance to 1 or more of the 7 antimicrobial classes included in the CMV2AGNF panel (18)] between 2003 and 2004 OBFS and the 2015 CIPARS pre-harvest farm isolates were also determined. Temporal changes in the use of antimicrobials administered via injection such as TIOinj, GEN (GENinj) and lincomycin-spectinomycin (LSinj), and trimethoprim-sulfadiazine administered via feed (TMSfed) were determined using the same logistic regression models described above.

Limitations of this present analysis

Detailed risk factor analysis for *Salmonella*- and *Campylobacter*-positive flock or sample status (OBFS, CIPARS broiler farm program) and risk factor analysis for resistance to each of the antimicrobials tested (e.g., AMU-AMR associations) are beyond the scope of this paper but were described in detail elsewhere (17) and in future analysis.

### Results

**Salmonella** (*n* = 692) from the 2003 to 2004 OBFS

A total of 150 chick placement *Salmonella* isolates from 38 flocks and 542 pre-harvest *Salmonella* isolates from 49 flocks had associated sampling information and barn identifiers, and, therefore, were susceptibility tested. At chick placement, S. Heidelberg was the most frequently isolated serovar from *Salmonella* recovered from meconium and cloacal swab specimens (44%, 38/87 isolates) and the barn environment *Salmonella* isolates (65%, 41/63). Similarly, at pre-harvest, S. Heidelberg was the most frequently isolated serovar from fecal samples or litter (40%, 170/421) and the barn environment *Salmonella* isolates (47%, 57/121). There were other non-typhoidal *Salmonella* serovars, with public health significance (e.g., S. Enteritidis, S. Infantis, S. Newport, S. Thompson, and S. Typhimurium) detected but at relatively lower proportions (<1% to 5%). The total numbers of unique serovars identified were similar in both farm sampling visits (chick placement, *n* = 16 serovars; pre-harvest, *n* = 17 serovars).

Table 1 summarizes the results of susceptibility testing of *Salmonella* isolates to individual antimicrobials. Between chick placement and pre-harvest visits, prevalence of resistance significantly ($P \leq 0.05$) increased among beta-lactam antimicrobials: amoxicillin-clavulanic acid (AMC), TIO, CRO, and cefoxitin (FOX) but remained stable for ampicillin (AMP; Table 1). *Salmonella* Heidelberg accounted for the majority of the TIO-R *Salmonella* isolates observed at chick
Amoxicillin-clavulanic acid (AMC) & 9 (4–21) & 16 (9–27)\textsuperscript{a} & 17 (10–29) & 24 (16–34)\textsuperscript{a,\textdagger} \\
Ceftiofur (TIO) & 9 (4–21) & 15 (8–27)\textsuperscript{a} & 13 (6–24) & 22 (14–31)\textsuperscript{a,\textdagger} \\
Ceftiximine (CRO) & 9 (4–21) & 15 (8–27)\textsuperscript{a} & 15 (8–27) & 22 (14–32)\textsuperscript{a,\textdagger} \\
Ciprofloxacin (CIP) & ND & ND & ND & ND \\
Ampicillin (AMP) & 27 (17–41) & 27 (18–39) & 32 (22–45) & 38 (29–47) \\
Azithromycin (AZI) & ND & ND & ND & ND \\
Cefotaxin (FOX) & 9 (4–21) & 15 (8–27)\textsuperscript{a} & 15 (8–27) & 22 (14–31) \\
Gentamicin (GEN) & 5 (2–12) & 4 (1–9) & 13 (6–27) & 7 (4–13)\textsuperscript{a} \\
Nalidixic acid (NAL) & 0 (0–5) & 0 (0–1) & 4 (1–14) & 0 (0–4) \\
Streptomycin (STR) & 21 (12–34) & 17 (9–28)\textsuperscript{a} & 23 (16–32) & 33 (24–43)\textsuperscript{a} \\
Trimepramethoxazole (TMSm) & ND & ND & 2 (0–11) & 3 (1–9) \\
Chloramphenicol (CHL) & 3 (1–10) & 3 (1–11) & 3 (1–9) & 3 (1–9) \\
Sulfisoxazole (SSS) & 11 (5–23) & 6 (3–13) & 23 (14–36) & 18 (11–26) \\
Tetracycline (TET) & 19 (10–33) & 13 (7–25)\textsuperscript{a} & 42 (30–54) & 53 (42–64)\textsuperscript{a} \\

\textsuperscript{a} Significant (P ≤ 0.05) farm-level sampling stage difference; arrows indicate the direction of the change between chick placement and pre-harvest sampling. 

The percent positive values were adjusted to account for multiple samples collected per farm. LCL — lower confidence limits; HCL — upper confidence limits; ND — resistant to the antimicrobial was not detected in all of the isolates tested.

Salmonella spp.

ESCHERICHIA COLI (GENERIC) (N = 271) FROM THE 2003 TO 2004 OBFS

Antimicrobial resistance among E. coli isolated from pooled cloacal/chick pad specimens from chick placement visits (n = 118; 39 flocks) and pooled fecal samples from pre-harvest visits (n = 153; 50 flocks) are summarized in Table I. Between chick placement and pre-harvest, resistance to the beta-lactam antimicrobials significantly increased (e.g., 13% to 22%, P ≤ 0.05; Table I). In contrast, GEN-R E. coli significantly decreased from chick placement (13%) to pre-harvest (7%, P ≤ 0.05; Table II). Consistent with the Salmonella results, there were no CIP-R or NAL-R isolates. Resistance to other antimicrobials was low (2% TMSm, 3% CHL), moderate (23% for STR, SSS), and high (43% for TET). When the prevalence of resistance among chick placement isolates was compared to pre-harvest isolates, the change in prevalence significantly increased (P ≤ 0.05) for STR and TET (Table I).

Campylobacter (n = 302) FROM THE 2003 TO 2004 OBFS

There was only 1 Campylobacter isolate isolated at a chick placement visit from a water sample from the drinker line. This isolate was susceptible to all 9 antimicrobials tested. The pre-harvest fecal isolates that originated from 22 flocks consisted of C. jejuni (87%, 262/302) and E. coli (13%, 40/302). Infrequent (<7%) resistance for each of telithromycin (TEL), AZM, clindamycin (CLI), and erythromycin (ERY) was noted. There were no isolates resistant to CIP, GEN, NAL, or florfenicol (FLR). The most frequent resistance was to TET at 54%.

Salmonella prevalence and serovar distribution comparing 2003 to 2004 OBFS and CIPARS farm, abattoir, and retail surveillance

Prevalence of Salmonella positive flocks rather than prevalence of Salmonella positive samples was reported in the OBFS (Table II) in which a flock was considered positive if at least one of the samples collected tested positive by bacterial isolation (17). In the CIPARS farm data, the percentage of positive samples in 2013 to 2015 ranged from 24% to 54% (Table II). At the abattoir, the percentage of Salmonella positive samples ranged from 15% to 35% (peaked in 2008) and at retail, it ranged from 17% to 54%. Overall, prevalence
of positive samples in both abattoir and retail sampling appeared to decrease.

**Farm** — Figure 1a summarizes the temporal variations in prevalence of selected serovars among total *Salmonella*. Serovars that contributed to ≤ 2% of all isolates were not included in the figure. From 2003 to 2004, in the OBFS S. Heidelberg was the most frequently isolated serovar, followed by S. Kentucky. There were no farm level data between 2005 and 2012, but in 2013, when the CIPARS broiler farm program was initiated, S. Kentucky was the most prevalent serovar isolated followed by S. Heidelberg. This ranking was unchanged in 2014, but in 2015, the ranking shifted with S. Heidelberg moving back into the number 1 ranking serovar.

**Abattoir**. Similar to the 2003 to 2004 OBFS, in 2003 the top 2 serovars detected at abattoir for CIPARS samples were *S. Heidelberg* followed by *S. Kentucky*. There were no farm level data between 2005 and 2012, but in 2013, when the CIPARS broiler farm program was initiated, S. Kentucky was the most prevalent serovar isolated followed by S. Heidelberg. This ranking was unchanged in 2014, but in 2015, the ranking shifted with S. Heidelberg moving back into the number 1 ranking serovar.

### Table II. Recovery of *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. from broiler chickens in the Ontario broiler farm study and on-farm, abattoir and retail CIPARS program.

<table>
<thead>
<tr>
<th>Stage of sampling</th>
<th>Year</th>
<th>% Positive/total samples or farms</th>
<th>% Positive/total samples or farms</th>
<th>% Positive/total samples or farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBFS, chick placement</td>
<td>2003/04</td>
<td>NA/NA 49% 44/90 farms a</td>
<td>2% 2/90 farms a</td>
<td>42/90 farms a</td>
</tr>
<tr>
<td>OBFS, pre-harvest</td>
<td>2003/04</td>
<td>NA/NA 69% 64/90 farms a</td>
<td>26% 24/90 farms a</td>
<td>18/90 farms a</td>
</tr>
<tr>
<td>Farm, chick placement</td>
<td>2013 85% 64/75</td>
<td>17% 13/75 b</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Farm, pre-harvest</td>
<td>2013 100% 120/120</td>
<td>54% 65/120 c</td>
<td>17% 20/120</td>
<td>16/120</td>
</tr>
<tr>
<td>Farm, chick placement</td>
<td>2014 87% 65/75</td>
<td>3% 2/75 b</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Farm, pre-harvest</td>
<td>2014 99% 166/168</td>
<td>25% 42/168 c</td>
<td>21% 35/168</td>
<td>20/168</td>
</tr>
<tr>
<td>Farm, chick placement</td>
<td>2015 88% 66/75</td>
<td>9% 7/75 b</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Farm, pre-harvest</td>
<td>2015 99% 195/196</td>
<td>54% 106/196 c</td>
<td>18% 36/196</td>
<td>20/196</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2003 97% 182/187</td>
<td>21% 66/136</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2004 99% 178/179</td>
<td>22% 60/271</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2005 100% 216/217</td>
<td>20% 57/282</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2006 98% 58/59</td>
<td>31% 91/296</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2007 98% 61/62</td>
<td>28% 90/317</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2008 98% 63/64</td>
<td>35% 110/314</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2009 100% 66/66</td>
<td>31% 99/320</td>
<td>14% 7/50</td>
<td>18/50</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2010 98% 50/51</td>
<td>29% 72/246</td>
<td>16% 40/246</td>
<td>18/246</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2011 100% 52/52</td>
<td>24% 50/211</td>
<td>14% 29/211</td>
<td>16/211</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2012 100% 51/51</td>
<td>27% 55/205</td>
<td>20% 41/206</td>
<td>18/206</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2013 100% 57/57</td>
<td>25% 59/240 c</td>
<td>18% 43/235</td>
<td>16/235</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2014 100% 58/58</td>
<td>15% 33/226 c</td>
<td>27% 61/226</td>
<td>18% 42/226</td>
</tr>
<tr>
<td>Abattoir</td>
<td>2015 98% 53/54</td>
<td>15% 35/236 c</td>
<td>18% 42/237</td>
<td>18% 42/237</td>
</tr>
<tr>
<td>Retail</td>
<td>2003 95% 137/144</td>
<td>16% 27/167</td>
<td>47% 78/166</td>
<td>37% 43/166</td>
</tr>
<tr>
<td>Retail</td>
<td>2004 95% 150/158</td>
<td>17% 54/315</td>
<td>45% 143/315</td>
<td>39% 117/315</td>
</tr>
<tr>
<td>Retail</td>
<td>2005 95% 145/153</td>
<td>9% 26/303</td>
<td>40% 120/303</td>
<td>34% 92/303</td>
</tr>
<tr>
<td>Retail</td>
<td>2006 97% 152/156</td>
<td>12% 36/311</td>
<td>34% 104/311</td>
<td>30% 90/311</td>
</tr>
<tr>
<td>Retail</td>
<td>2007b 98% 157/161</td>
<td>54% 172/320</td>
<td>37% 117/320</td>
<td>31% 101/320</td>
</tr>
<tr>
<td>Retail</td>
<td>2008 96% 150/156</td>
<td>45% 139/311</td>
<td>39% 121/311</td>
<td>30% 101/311</td>
</tr>
<tr>
<td>Retail</td>
<td>2009 95% 155/164</td>
<td>43% 142/328</td>
<td>31% 101/328</td>
<td>27% 90/328</td>
</tr>
<tr>
<td>Retail</td>
<td>2010 86% 100/116</td>
<td>39% 90/232</td>
<td>28% 64/232</td>
<td>25% 55/232</td>
</tr>
<tr>
<td>Retail</td>
<td>2011 93% 137/147</td>
<td>40% 119/294</td>
<td>24% 71/293</td>
<td>22% 60/293</td>
</tr>
<tr>
<td>Retail</td>
<td>2012 92% 107/116</td>
<td>44% 102/232</td>
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<td>35% 78/232</td>
</tr>
<tr>
<td>Retail</td>
<td>2013 93% 110/118</td>
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<td>35% 83/231</td>
<td>35% 78/231</td>
</tr>
<tr>
<td>Retail</td>
<td>2014 92% 144/157</td>
<td>24% 75/312 c</td>
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<td>25% 78/312</td>
</tr>
<tr>
<td>Retail</td>
<td>2015 91% 69/76</td>
<td>17% 26/151 c</td>
<td>26% 40/151</td>
<td>26% 40/151</td>
</tr>
</tbody>
</table>

---

*a* In this study a flock was deemed positive if at least one of the environmental and bird samples collected tested positive.

*b* Methodological enhancement to the recovery of *Salmonella* was initiated.

*c* Samples were from progenies of Ontario broiler breeders vaccinated with *Salmonella*.


NA — Not available.
Figure 1. Temporal variations in percentage of total *Salmonella* for 5 *Salmonella* serovars, farm to retail, from 2003 to 2015. Arrows signify the initiation of an enhanced food safety programs including *Salmonella* vaccination protocol in broiler breeder flocks; thus, most of the chicks placed in sentinel farms surveyed by CIPARS (2013–2015) were from vaccinated breeders. Methodological enhancement to the recovery of *Salmonella* was initiated in 2007 (18).

### a. Farm

<table>
<thead>
<tr>
<th>Year</th>
<th>Typhimurium</th>
<th>Kentucky</th>
<th>Infantis</th>
<th>Heidelberg</th>
<th>Enteritidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003-04</td>
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</tr>
<tr>
<td>2005</td>
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<td>54%</td>
<td>0%</td>
<td>32%</td>
<td>3%</td>
</tr>
<tr>
<td>2006</td>
<td>0%</td>
<td>2%</td>
<td>0%</td>
<td>21%</td>
<td>0%</td>
</tr>
<tr>
<td>2007</td>
<td>0%</td>
<td>5%</td>
<td>0%</td>
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</tr>
<tr>
<td>2008</td>
<td>0%</td>
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</tr>
<tr>
<td>2009</td>
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</tr>
<tr>
<td>2010</td>
<td>0%</td>
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</tr>
<tr>
<td>2011</td>
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<td>49%</td>
<td>0%</td>
<td>15%</td>
<td>0%</td>
</tr>
<tr>
<td>2012</td>
<td>0%</td>
<td>49%</td>
<td>0%</td>
<td>25%</td>
<td>0%</td>
</tr>
<tr>
<td>2013</td>
<td>0%</td>
<td>15%</td>
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</tr>
<tr>
<td>2014</td>
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<td>22%</td>
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</tr>
<tr>
<td>2015</td>
<td>0%</td>
<td>14%</td>
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<td>0%</td>
</tr>
</tbody>
</table>

### b. Abattoir

<table>
<thead>
<tr>
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<th>Typhimurium</th>
<th>Kentucky</th>
<th>Infantis</th>
<th>Heidelberg</th>
<th>Enteritidis</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
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<td>14%</td>
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<td>3%</td>
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</tbody>
</table>

### c. Retail

<table>
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<th>Heidelberg</th>
<th>Enteritidis</th>
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<tr>
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<td>15%</td>
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<tr>
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<td>15%</td>
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<td>31%</td>
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</tr>
</tbody>
</table>
Salmonella

In 2003, S. Heidelberg (70%) and S. Kentucky (11%) were the top 2 serovars isolated from retail chicken, which is consistent with the 2003 to 2004 OBFS and abattoir surveillance (Figure 1c). Salmonella Heidelberg remained in the top position, followed by S. Kentucky, until 2006 when similar to abattoir, S. Kentucky prevalence surpassed S. Heidelberg. This order was maintained until 2012...
when these 2 serovars again flipped and S. Heidelberg moved back to the top position. Salmonella Heidelberg has remained the most common serovar since 2012 (Figure 1c).

Escherichia coli and Campylobacter spp. prevalence. Recovery rates were relatively stable across all CIPARS surveillance components (Table II).

Data integration: Farm, abattoir, and retail surveillance and AMU

Ceftiofur. Figure 2 summarizes resistance to TIO among Salmonella and E. coli from Ontario broiler chicken samples collected in multiple CIPARS surveillance components and the OBFS. Ceftiofur resistance in isolates from farm, abattoir, and retail samples fluctuated over time. Trends in TIO-R Salmonella at abattoir and retail show an initial peak in 2004 to 2005, followed by decline to 2008, then increase to 2011, then gradual decline to 2015. Pre-harvest farm TIO-R Salmonella increased from 15% in 2003 to 2004 to 43% in 2013 (first CIPARS surveillance year on farm), then declined to 8% in 2015. Comparing point estimates from the start (2003 to 2004) and end (2015) of the observation period, TIO-R Salmonella was similar among retail isolates (12%), increased among abattoir isolates (2003: 3%, 2015: 14%) and modestly decreased among farm isolates (2003 to 2004:
15%, 2015: 8%). In 2015, the prevalence of TIO resistance was most common among S. Heidelberg from chickens at pre-harvest farm (89%, 8/9), abattoir (20%, 1/5), and retail (67%, 2/3). Other TIO resistant serovars included S. Typhimurium (1 farm isolate, 2 abattoir isolates), S. Infantis (1 retail isolate), S. Kentucky (1 abattoir isolate), and S. Schwarzengrund (1 abattoir isolate). Unlike in 2003 to 2004, none of the more recent S. Kentucky isolates from CIPARS farm or retail surveillance exhibited resistance to TIO.

Among E. coli (Figure 2), TIO-R prevalence fluctuated over time in all surveillance components with a gradual increase in abattoir and retail isolates from 2010 to 2011 followed by a general decline to 2015. In pre-harvest farm isolates, there was a significant (P ≤ 0.05) drop in TIO-R E. coli from 22% in 2003 to 2004 to 6% in 2015; among placement farm isolates there was an increase from 13% in 2003 to 2004 to 22% in 2015.

Antimicrobial use information was unavailable prior to the initiation of the CIPARS farm surveillance program, but in 2013 to 2015, there were no broiler producers that reported TIO or any third-generation cephalosporins at the hatchery.

Gentamicin. GEN-R Salmonella was generally low (< 10%) in all CIPARS surveillance components and the OBFS (Figure 3). However, among E. coli, there was an increase; GEN-R E. coli isolates were detected from farm samples, with a modestly increased trend across all surveillance components between 2003 and 2015. The only significant (P ≤ 0.05) increase in GEN-R E. coli was seen at retail with an increase from 7% to 22% between 2003 and 2015.

Through the CIPARS farm program, Ontario broiler producers occasionally (2.5%, 3/121 flocks surveyed in 2013 to 2015) reported the use of aminoglycoside antimicrobials (i.e., the same class as GEN) (e.g., apramycin, neomycin). The reported use of a related...
antimicrobial, spectinomycin, an aminocyclitol, combined with lincomycin, a lincosamide (LSinj), though not significant, increased from 17% to 29% of the broiler producers in Ontario participating in the CIPARS farm program (Figure 3).

**Trimethoprim-sulfadiazine.** Similar to GEN, an overall increasing trend in TMSm-R Salmonella was noted across all surveillance components. There was a significant ($P \leq 0.05$) increase in TMSm-R E. coli at pre-harvest farm samples from 3% to 23% (Figure 4). Between sampling visits (chick placement and pre-harvest), resistance prevalence for TMSm increased in contrast to GEN, where prevalence consistently decreased between sampling visits from 2013 to 2015 surveillance years and in 2003 to 2004 ($P \leq 0.05$) as previously described (Table I).

Trimethoprim-sulfadiazine administered via feed (TMSfeed) was reported by 21% to 23% (no significant temporal change noted) of broiler producers in Ontario participating in the CIPARS Farm program between 2013 and 2015.

**Campylobacter from the 2003 to 2004 OBFS compared to CIPARS Ontario farm, pre-harvest isolates**

In 2003, CIP-R Campylobacter was detected in 4% of the retail isolates but no resistant isolates were detected in pre-harvest farm isolates obtained from the 2003 to 2004 OBFS (Figure 5). At the abattoir (Campylobacter testing started in 2010), CIP-R Campylobacter was first detected in 2011. Figure 5 shows a trend toward increasing CIP-R prevalence; however, a significant difference was noted only for retail (4% to 15%, $P \leq 0.05$).

No producers reported the use of fluoroquinolones [enrofloxacin (ENR) and danofloxacin, veterinary fluoroquinolones available for use in food animals in Canada, but not labelled for use in broiler chickens] in the CIPARS Ontario flocks surveyed between 2013 and 2015.

**Multiclass resistance from the 2003 to 2004 OBFS compared to CIPARS Ontario farm, pre-harvest isolates**

As summarized in Table III, resistance in pre-harvest farm Salmonella isolates shifted between 2003 and 2004 and 2015. There was a 6% increase in the proportion of susceptible isolates and 10% increase ($P \leq 0.05$) in isolates resistant to one class. These corresponded to a decrease in 2 to 3 classes (6% decrease), 4 to 5 classes (7% decrease), and 6 to 7 classes (3% decrease). Among E. coli, results were different from those observed for Salmonella. Between 2003 and 2004 and 2015, the percentage of isolates increased in 2 multiclass resistance categories (2 to 3 classes: 9% increase and 4 to 5 classes: 4% increase), and decreased in 2 other resistance categories: (susceptible to all: 7% decrease and 1 to 2 classes: 5% decrease). Campylobacter isolates also showed a shift in the proportion of susceptible isolates. Of importance to note is the emergence of isolates exhibiting the CIP-NAL-TET multidrug resistance pattern (19% of total isolates). These isolates contributed to the overall increase in the proportion of isolates that were resistant to 2 to 3 classes of antimicrobials (27% increase between 2003 to 2004 and 2015, $P \leq 0.05$). In the same timeframe, there was also a decrease in the percentage of isolates that were susceptible to all antimicrobials tested (17% decrease) and the percentage of isolates resistant to the 1 to 2 classes (10% decrease).

**Discussion**

Our study assessed AMR prevalence among Salmonella, E. coli, and Campylobacter isolates from Ontario broiler chicken farms from 2003 to 2004; because of study design similarities to subsequent CIPARS farm-level surveillance, these results were considered as baseline farm-level AMR data and complemented early CIPARS abattoir and retail results. This paper also serves as a valuable
reference point, in light of upcoming industry and federal legislations concerning AMU to reduce AMR threats in both animals and humans in Canada (11,12,19). Overall, sampling frame and design varied between farm, abattoir, and retail surveillance. However, the sampling methodology was designed to generate data representative of Ontario broiler chicken farms (farms were selected based on certain inclusion and exclusion criteria, and distributed geographically throughout Ontario (17,18)), slaughtered chickens (chickens were sampled proportional to slaughter volume from all federally registered abattoirs in Ontario) and retail meats (chicken meat sampled from randomly selected geographic areas within Ontario selected based on population). Further, the microbiology methodology was consistent allowing the integration of these surveillance data, which is important in facilitating the detection of emerging food safety threats and assessing the efficacy of interventions.

The 2003 to 2004 OBFS identified several Salmonella serovars implicated in human infections in Canada including S. Enteritidis, S. Typhimurium, S. Heidelberg, S. Newport, S. Infantis, and S. Hadar in Ontario broiler chicken flocks. There was a gap (2004 to 2012) in farm-based surveillance for broiler chickens, but another enteric surveillance program, FoodNet Canada (formerly C-EnterNet), also detected these serovars in broiler chicken farms located within an Ontario sentinel site (Region of Waterloo, Ontario) between 2006 and 2011 (2). Our data combined with FoodNet Canada indicates that the relative proportion of these serovars shifted over time and in recent surveillance years, the prevalence of certain serovars, particularly those that exhibit resistance to antimicrobials (e.g., TIO-R S. Heidelberg) or implicated in food-borne outbreaks (e.g., S. Enteritidis) in Ontario has decreased in the broiler chicken population (20). The shift in serovar distribution may be a result of ongoing enhancements to farm food safety programs, e.g., vaccinations, monitoring (9). It is important to note that susceptible and antimicrobial-resistant Salmonella were detected from chick placement samples in both the OBFS and CIPARS farm programs. Although the re-use of litter is not practiced in Canadian broiler flocks, potential carry-over from previous flock or vertical-transmission may explain the recovery of Salmonella at chick placement. At the very minimum, dry-cleaning prior to chick placement is recommended and a full washing and disinfection are required once a year as per the Safe, Safer, Safest program (6).

During the 2003 surveillance year, TIO-R Salmonella was prevalent (3% to 15%) across all surveillance components. Hatchery AMU data are not available prior to 2003 because there were no farm-level information available elsewhere (e.g., flock sheets) and CIPARS farm surveillance was not yet operational, so it is unknown exactly when the hatcheries began using TIO inj in Ontario. Passive surveillance of clinical Salmonella isolates submitted to NML at Guelph between 1994 and 1999 detected TIO-R Salmonella in turkeys and cattle but not in chickens (21). In 2002, a study of retail meats originating from Ontario detected FOX and CRO resistant E. coli carrying beta-lactamase conferring gene, bla CMY-2 (22). Although no AMU information are available for that time for Ontario, a Quebec study conducted during the same timeframe (2003 to 2004), reported in ovo TIO inj in 76% of the broiler flocks sampled (4). Quebec hatcheries implemented a voluntary withdrawal of TIO inj in 2005 to 2006 that resulted in a rapid drop in TIO-R S. Heidelberg from retail chicken (62% to 7%; P < 0.001) (5). In 2007 the Quebec poultry industry partially reinstituted the use of TIO inj, which may explain the re-emergence of TIO-R S. Heidelberg in that province (5). Ontario retail chicken data showed a similar TIO-R Salmonella trend, although there was no hatchery or farm-level data to characterize TIO inj use reductions. The CIPARS broiler farm surveillance data for Ontario between 2013 and 2015 indicates no reported TIO inj at the hatchery level. As previously described, the poultry industry formally eliminated the preventive use of antimicrobials considered of very high importance to human medicine (including third generation cephalosporins) in broiler chickens and turkeys in May 2014. Anecdotal information from the poultry industry suggests that some hatcheries started reducing or eliminating the TIO inj as early as 2010, and although there is no AMU data to corroborate this, the fact that none of the CIPARS flocks in Ontario reported any TIO inj use at the hatchery in 2013 is consistent with a possible earlier AMU practices change. Historically (2003 to 2013 CIPARS data), a high proportion of S. Heidelberg and S. Kentucky chicken isolates exhibited resistance...
to TIO (20). The decreasing proportion of S. Heidelberg/TIO-R S. Heidelberg isolates and increasing proportion of S. Kentucky/TIO-susceptible S. Kentucky isolates may also explain the shift towards susceptibility to TIO among Salmonella spp. overall. The more recent S. Heidelberg prevalence and their AMR profile are similar to historic data (23,24). Overall, decreasing prevalence of TIO-R isolates is indicative of the impact of AMU and food safety interventions against Salmonella in Ontario broiler chickens but emerging GEN-R and TMS-R isolates will need to be monitored.

Surveillance of E. coli, monitored as an indicator organism, showed similar results to Salmonella for resistance to TIO. As for Salmonella, [FOX resistant and carried the bla\textsubscript{CMY2} plasmid (25)] the TIO-R E. coli isolates from this present study exhibited an AMC-TIO-FOX-CRO resistance pattern, consistent with isolates harboring the bla\textsubscript{CMY2} gene, a plasmid-mediated AmpC beta-lactamase conferring gene in E. coli (26) found in Canadian (25) and American chickens (27). The use of TIO\textsubscript{inj} in the early stages of the bird’s life has been associated with the emergence of TIO-R E. coli that can persist throughout the life of the broiler flock or throughout the production period for laying hens (28,29). As previously described, the high proportion of chicks/hatching eggs medicated with TIO\textsubscript{inj} in Quebec hatcheries in 2003 to 2004 was significantly associated with the detection of TIO-R E. coli in broiler chickens (4). The voluntary withdrawal resulted in a rapid drop in TIO-R E. coli retail isolates (34% to 6%; P < 0.0001), in addition to a drop in TIO-R S. Heidelberg as previously described (5). Ceftiofur resistance among E. coli showed a decreasing trend and reached ≤ 2003 levels in 2015. The CIPARS farm program continues to detect TIO-R E. coli from chick pads/meconium and in pre-harvest broilers although there is no reported TIO\textsubscript{inj} use at the hatcheries in Ontario. In Japan, there was a gradual decrease in cefazolin and cefotaxime/TIO resistance 2 y after the withdrawal of TIO\textsubscript{inj} use in hatcheries (30). The less frequent resistance observed in the Canadian isolates may be suggestive of a transitional period in gut bacteria and the environment (31), such as a shift from highly resistant to susceptible gut flora. Other potential contributing factors may include contamination from the upper levels of the production pyramid, maintenance of resistant strains in the environment (e.g., litter was disposed but no cleaning and disinfection practiced between flock cycles), the use of related antimicrobials (e.g., aminopenicillins), and international/provincial movement of poultry products. The finding of beta-lactam resistant E. coli in CIPARS chick placement samples is suggestive of vertical transmission of beta-lactam resistant strains or genes/plasmid transfer from upper levels of production as described in the literature (32–34). As evidenced by the presence of resistant isolates found on barn environmental surface swabs in CIPARS farm surveillance and in other similar studies (35,36), beta-lactam resistant E. coli are being maintained in the environment and, therefore, can act as a reservoir for resistant genes/plasmids. These findings highlight the importance of proper cleaning, disinfection, and down-time or rest periods between flocks in order to reduce potential carry-over to newly placed chicks.

Another notable observation was an increase in GEN-R E. coli. This trend in GEN resistance appears consistent with the reported use of GEN\textsubscript{inj}, or a related antimicrobial, spectinomycin. In Quebec, the trend in GEN-R prevalence among clinical E. coli isolates corresponded with LS\textsubscript{inj} use in broiler production. Molecular characterization of aminoglycoside and aminocyclitol genes also indicated that the use of this drug can co-select for GEN resistance (37). Trimethoprim-sulfamethoxazole resistance prevalence was also identified as an emerging trend. Similar to the trend observed for GEN resistance, TMSm-R increase corresponded with the reported use of TMS\textsubscript{feed}. Although this antimicrobial drug combination is not labelled for use in broilers, in the CIPARS farm surveillance program, it was reportedly used by broiler producers for the treatment of respiratory and septicemic diseases in neonatal and growing broilers. The emergence of TMSm-R in Ontario broiler chicken E. coli isolates is potentially due to combination of co-selection by sulfonamides (i.e., exposure to sulfadiazine present in TMS\textsubscript{feed} or sulfamethoxazole and sulfamethoxazole via water) and trimethoprim (exposure to trimethoprim present in TMS\textsubscript{feed}) (38). Because of these emerging AMR trends, the level of on-farm use requires ongoing monitoring. The association between use at the hatchery and farm and other hatchery/farm level risk factors collected through the CIPARS farm questionnaire needs to be evaluated.

Depending on the antimicrobial and the timeframe, there was a shift in the prevalence of resistance among E. coli detected between chick placement and pre-harvest. Factors that could influence this shift include antimicrobial use/selection pressure in the industry overall and specifically during the broiler grow-out period, the bird’s intestinal microbiome (39,40), and the microbiome’s interaction with multiple factors such as host immunity and diet (39,40). The use of multiple antimicrobials concurrently or successively throughout the grow-out period has been shown to result in the development of resistant isolates with complex multiclass resistance patterns that could subsequently displace susceptible microflora (41,42). This may explain the shift towards resistance to multiple classes of antimicrobials among E. coli. Thus, careful consideration of treatment options and frequency of use are important in reducing overall AMR in targeted bacteria in broiler chickens. Further molecular investigation is also warranted to better understand the dissemination and transfer of resistance, co-selection and cross-selection properties as they pertain to transferrable mobile genetic elements, such as plasmids.

Campylobacter, one of the major causes of foodborne infections in humans, which is a reportable disease in Ontario (43), has been regularly detected from Ontario chicken and chicken meats. Ciprofloxacin resistant Campylobacter was first observed by CIPARS in the western provinces in 2007 and peaked in 2009 (16), but this study found that resistant strains are now relatively common in Ontario broiler chickens. With no reported use of florquinolones at the farm level in Canada, it is unclear how CIP-R Campylobacter emerged in Ontario broiler chicken flocks. The use of ENR was not captured by the CIPARS farm program because of the surveillance timeframe or that usage was relatively low (i.e., therapeutic/metaphylactic use for infections unresponsive to currently available drugs). Potential sources such as cross-contamination from other production animals or wildlife colonized by CIP-R C. jejuni strains, and other potential farm-level sources, as identified in a recent systematic review (44) should be considered in future research. Floruquinolone-resistant Campylobacter in broiler flocks has been shown to persist after the cessation of floruquinolone use in the poultry industry and may

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require additional interventions to reduce the prevalence in the poultry production chain (45,46).

In summary, resistance profiles and Salmonella serovar distribution in broiler chickens in Ontario have shifted over time; towards increased prevalence of resistance to GEN, TMS, CIP, and decreased prevalence of resistance to TIO. The continued decreasing resistance trend to beta-lactams among Salmonella and E. coli isolates suggests that reduction in resistance can, in some cases, occur relatively quickly after the introduction of an AMU reduction policy. Additionally, the changes in the proportional prevalence of Salmonella serovars with public health significance (e.g., decrease in S. Heidelberg, S. Enteritidis, S. Typhimurium) is consistent with the idea that pathogen control programs, such as vaccination in upper levels of the production pyramid and food safety enhancement throughout the food chain, are working. However, the increase in resistance to GEN and TMSm in E. coli and Salmonella and CIP resistance in Campylobacter may signal emerging trends, and warrants ongoing monitoring and consideration with respect to prudent use practices. The reason for the increasing trend in CIP-R Campylobacter is unknown. This study highlights the importance of integrated surveillance in detecting emerging threats and determining the efficacy of interventions. The data also serve as a valuable reference point in light of upcoming changes in policy for AMU in animals in Canada.

Acknowledgments

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References


Pharmacokinetics of cannabidiol administered by 3 delivery methods at 2 different dosages to healthy dogs


Abstract

The purpose of this study was to determine the pharmacokinetics of cannabidiol (CBD) in healthy dogs. Thirty, healthy research dogs were assigned to receive 1 of 3 formulations (oral microencapsulated oil beads, oral CBD-infused oil, or CBD-infused transdermal cream), at a dose of 75 mg or 150 mg q12h for 6 wk. Serial cannabidiol plasma concentrations were measured over the first 12 h and repeated at 2, 4, and 6 wk. Higher systemic exposures were observed with the oral CBD-infused oil formulation and the half-life after a 75-mg and 150-mg dose was 199.7 ± 55.9 and 127.5 ± 32.2 min, respectively. Exposure is dose-proportional and the oral CBD-infused oil provides the most favorable pharmacokinetic profile.

Résumé

Le but de la présente étude était de déterminer la pharmacocinétique du cannabidiol (CBD) chez des chiens en santé. Trente chiens de recherche en santé ont été assignés à recevoir une des trois formulations (de l’huile micro-encapsulé dans des billes par voie orale, de l’huile infusé de CBD par voie orale, ou une crème infusé de CBD par voie transdermique), à une dose de 75 mg ou 150 mg q12h pendant 6 semaines. Les concentrations plasmatiques de cannabidiol ont été mesurées pendant les 12 premières heures et répétées après 2, 4 et 6 semaines. Les expositions systémiques les plus élevées ont été observées avec la formulation d’huile infusé de CBD administrée par voie orale et la demi-vie après une dose de 75 mg et de 150 mg était de 199,7 ± 55,9 et 127,5 ± 32,2 min, respectivement. L’exposition est proportionnelle à la dose et l’huile infusée de CBD par voie orale fournit le profile pharmacocinétique le plus favorable.

(Crutdut par Docteur Serge Messier)

Introduction

Cannabis has been used for centuries in human medicine for both recreational and medicinal purposes. In human medicine, cannabis-based extracts have been used for the treatment of spasticity, central pain, lower urinary tract symptoms, spastic dysfunctions, peripheral neuropathic pain, brachial plexus avulsion symptoms, nausea and vomiting associated with cancer chemotherapy, loss of appetite, rheumatoid arthritis, intractable cancer pain, spinal cord injuries, Tourette's syndrome, psychoses, epilepsy, glaucoma, Parkinson’s disease, and dystonia (1–4). Although there are anecdotal success stories for treating many of the same diseases in pets, no scientific reports have been published to date (1,5–7).

If cannabidiol (CBD) is shown to be measurable in canine plasma, further studies investigating the efficacy of CBD for various diseases, including chronic pain, neuropathic pain, epilepsy, appetite stimulation, and anxiety, could be considered.

The chemical substances isolated from Cannabis sativa, phyto-cannabinoids, are divided into the psychotropic group and non-psychotropic group (5). The major psychoactive constituent of C. sativa, delta-9-tetrahydrocannabinol (Δ9-THC), causes toxicosis in dogs and is therefore of limited use in canine patients (8,9). The list of non-psychotropic compounds is expanding, but cannabidiol (CBD) is the most promising phytocannabinoid candidate, owing to its non-psychotropic effects, low toxicity, and high tolerability (10–13).

The purpose of this study was to determine the pharmacokinetics of orally and transdermally administered CBD and to compare the CBD plasma concentrations of 3 different delivery methods at 2 different dosages. We present a 3-part hypothesis: i) a single dose of CBD will result in measurable blood levels within 12 h; ii) daily administration of CBD will result in sustained blood levels; and iii) topical formulations for CBD delivery will have higher blood levels because of the elimination of the hepatic first-pass effect.

Materials and methods

Dogs

This study was carried out under the strict regulations of the Institutional Animal Care and Use Committee. All aspects of this study were approved by Colorado State University’s Institutional Animal Care and Use Committee (protocol ID: 15-5782A, approved: February 19, 2016). A power calculation was conducted, which showed that 10 subjects in each dose group would achieve a statistical power of 80% with a minimum Cmax (maximum concentration)
difference of 200 ng/mL between the groups with a standard deviation of 100 ng/mL. Thirty-one healthy adult, sexually intact male, purpose-bred research beagle dogs, from 4 to 5 y of age, weighing an average of 13 kg (9.5 to 16.2 kg) were evaluated. Upon arrival, the dogs were determined to be healthy through physical examinations carried out by either a Board-certified neurologist or a neurology resident and laboratory work, including complete blood (cell) count (CBC), chemistry panel, urinalysis, and pre- and postprandial bile acid assay. Animals were excluded if there was a comorbidity with a poor prognosis, abnormalities on blood work, or if they were currently receiving medications. Thirty dogs met the inclusion criteria and were enrolled in the study; 1 dog was excluded based on abnormal blood work.

All animals were kept in an on-site research facility and were checked regularly for feeding, cleaning, and overall appearance. Each dog was housed in an individual run as space allowed; beyond that, they shared a run with 1 other compatible dog. All dogs were evaluated weekly by a veterinarian who conducted complete physical examinations, as well as twice-daily general health assessments by the animal care staff and veterinary students.

**Treatment and sample collection**

The CBD was provided and formulated by Applied Basic Science Corporation, a 3rd-party, contracted enterprise within the state of Colorado. A random number generator was used to randomly assign each dog to 1 of 3 CBD delivery methods as Group 1 (CBD-infused transdermal cream), Group 2 (oral microencapsulated oil beads), or Group 3 (oral CBD-infused oil). Dogs in all 3 groups were then further divided into 2 different dosing groups (5 dogs/group) in an open-label study, to receive either 75 mg q12h (subgroup a) or 150 mg q12h (subgroup b) (Table I). All dogs were therefore administered a total daily dose of either 150 or 300 mg of CBD using 1 of the following delivery methods: CBD-infused transdermal cream (110 mg/mL) applied to the pinnae; beads of microencapsulated oil in capsules (microencapsulated oil beads; 25-mg and 50-mg capsule sizes); or CBD-infused oil (75 mg/mL or 150 mg/mL). The 2 doses corresponded with approximately 10 mg/kg body weight (BW) per day or 20 mg/kg BW per day. For the duration of the study, all dogs were administered each dose of CBD after a small meal.

In the first part of this 2-part study, CBD pharmacokinetics were measured during the initial 12 h of dose administration. Before the start of the study and after a 12-hour fast, an indwelling jugular catheter was placed and maintained throughout the pharmacokinetic (PK) blood draws or until it became non-patent, dislodged, or there were signs of irritation, in which case blood was collected percutaneously. Blood sampling for CBD plasma concentrations (1.3 mL) occurred before CBD was administered (0 min) and at times 30, 60, 120, 240, 360, 480, 600, and 720 min, for a total of 9 sample points. Each sample was placed into a lithium heparin microtube and immediately set on ice.

In the second part of the study, all dogs continued receiving a total daily dose of either 75 mg or 150 mg q12h of their respective delivery method, for a total of 6 wk. At 2, 4, and 6 wk after the first

<table>
<thead>
<tr>
<th>Group (5 dogs/group)</th>
<th>Delivery method</th>
<th>Approximate dose (mg/kg body weight per day)</th>
<th>Dose (mg q12h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>CBD-infused transdermal cream</td>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>1b</td>
<td>CBD-infused transdermal cream</td>
<td>20</td>
<td>150</td>
</tr>
<tr>
<td>2a</td>
<td>Microencapsulated oil beads</td>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>2b</td>
<td>Microencapsulated oil beads</td>
<td>20</td>
<td>150</td>
</tr>
<tr>
<td>3a</td>
<td>CBD-infused oil</td>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>3b</td>
<td>CBD-infused oil</td>
<td>20</td>
<td>150</td>
</tr>
</tbody>
</table>

Figure 1. Single-dose cannabidiol (CBD) plasma concentration. The 12-hour, single-dose CBD plasma concentration (mean +/- standard deviation) at 2 different dosages (75 mg, top; 150 mg, bottom) for transdermal cream, microencapsulated oil beads, and CBD-infused oil.
Table II. Non-compartmental pharmacokinetic analysis of CBD plasma concentrations after a single dose of 75 mg or 150 mg using 3 different formulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>75 mg</th>
<th>150 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CBD-infused oil</td>
<td>CBD-infused transdermal cream</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>ng/mL</td>
<td>625.3 ± 164.3</td>
<td>74.3 ± 127.2</td>
</tr>
<tr>
<td>$C_{\text{max}}$/dose</td>
<td>ng/mL</td>
<td>110.1 ± 29.1</td>
<td>11.3 ± 18.9</td>
</tr>
<tr>
<td>$AUC_{0-\text{t}}$</td>
<td>min•μg/mL</td>
<td>135.6 ± 46.3</td>
<td>11.7 ± 18.9</td>
</tr>
<tr>
<td>$AUC_{0-\text{inf}}$</td>
<td>min•μg/mL</td>
<td>147.1 ± 94.9</td>
<td>ND</td>
</tr>
<tr>
<td>AUC % extrapolated</td>
<td></td>
<td>8.0 ± 3.0</td>
<td>ND</td>
</tr>
<tr>
<td>$AUC_{0-\text{inf}}$/dose</td>
<td>min•μg/mL</td>
<td>25.8 ± 8.6</td>
<td>ND</td>
</tr>
<tr>
<td>MRT</td>
<td>Min</td>
<td>217 ± 46</td>
<td>490 ± 74</td>
</tr>
<tr>
<td>$T_{1/2, a}$</td>
<td>Min</td>
<td>199.7 ± 55.9</td>
<td>127.5 ± 32.2</td>
</tr>
<tr>
<td>Relative %</td>
<td>100</td>
<td>70.1</td>
<td>8.6</td>
</tr>
</tbody>
</table>

*Expressed as harmonic mean with pseudo-standard deviation. All other parameters are expressed as mean with standard deviation. $C_{\text{max}}$ — maximal concentration; AUC — area under the curve; $C_{\text{max}}$/dose and $AUC_{0-\text{inf}}$/dose are the dose-normalized values for maximum plasma concentration and total exposure from time 0 to infinity, respectively; MRT — mean residence time; ND — not determined due to lack of elimination phase in the concentration-time profiles; $T_{1/2}$ — half-life.

dose, blood was collected for CBD plasma concentrations. Each blood sample for CBD plasma levels was centrifuged for 10 min at 2000 × g and 8°C. The plasma was separated from the red blood cells, placed in a cryotube, and stored at −80°C until analysis at the end of the 6-week study period. Samples were spun and frozen within 2 h of collection.

**Extraction of cannabidiol from plasma**

The Colorado State University Proteomics and Metabolomics Laboratory measured the CBD plasma concentrations from 30 beagle dogs over 12 time points.

Aliquots of plasma were stored at −80°C until time of extraction. For CBD extraction, plasma was thawed on ice and 50 μL of each sample was placed into a 2.0-mL glass extraction vial, kept chilled on ice. Two hundred microliters of cold (−20°C) 100% acetonitrile (spiked with 60 ng/mL of d3-CBD) was added to each sample and vortexed at room temperature for 5 min. Two hundred microliters of water were added and vortexed for an additional 5 min. One milliliter of 100% hexane was added to each sample and vortexed for a final 5 min. Phase separation was enhanced under centrifugation at 1000 × g for 15 min at 4°C. The upper hexane layer was transferred to newly labeled glass vials (~ 900 μL per sample), carefully avoiding the middle and lower layers. Samples were concentrated to dryness under nitrogen gas (N₂) and re-suspended in 60 μL of 100% acetonitrile.

**Standard curve**

Four, 10-point calibration curves of CBD were generated in matrix background using a pooled blank canine serum. Concentrations ranged from 1 ng/mL to 1600 ng/mL (2.5x dilution series). Fifty microliters of each fortified sample were extracted as described and 4 curves were generated to accommodate each day of data collection, as well as extraction day/batch.

**Data collection and analysis**

Liquid chromatography-mass spectrometry (LC-MS) was carried out on an Acquity UPLC (Waters, Milford, Massachusetts, USA) coupled to an Xevo TQ-S triple quadrupole mass spectrometer (Waters). Chromatographic separations were carried out on a Phenyl-Hexyl stationary phase column (1 × 100 mm, 1.8 μM, Waters). Mobile phases were 100% methanol (B) and 0.1% formic acid and water (A). The analytical gradient was as follows: time = 0 min, 0.1% B; time = 6 min, 97% B; time = 7.0 min, 97% B; time = 7.5 min, 0.1% B; time = 12.0 min, 0.1% B. Flow rate was 200 μL/min and injection volume was 2 μL. Samples were held at 4°C in the autosampler and the column was operated at 70°C. Samples were directly injected into the mass spectrometer, which was operated in selected reaction monitoring mode, in which a parent ion was selected by the 1st quadrupole fragmented in the collision cell, then a fragment ion selected by the 3rd quadrupole. Productions, collision energies, and cone voltages were optimized for CBD and d3-CBD by direct injection of individual synthetic Cerilliant analytical reference standards. Inter-channel delay was set to 5 ms. The LC-MS was conducted in positive ionization mode with the capillary voltage set to 3.2 kV. Source temperature was 150°C and desolvation temperature was 500°C. Desolvation gas flow was 1000 L/h, cone gas flow was 150 L/h, and collision gas flow was 0.2 mL/min. Nebulizer pressure was set to 7 bar. Argon was used as the collision gas; otherwise nitrogen was used.
**Calculation of CBD concentration in plasma**

All raw data files were imported into Skyline (MacCoss Lab, Department of Genome Sciences, University of Washington, Seattle, Washington) and peak areas extracted for CBD and d3-CBD. Quantitation of analyte in plasma samples was based on linear regression of calibration curves and extrapolation using the analyte peak area to internal standard peak area ratios. All calibration curves were linear over the range of concentrations tested ($r^2 > 0.998$). The limit of detection of the assay was 0.3 ng/mL and was calculated as the standard error divided by the slope of the linear regression of the calibration curves multiplied by 3.3. The limit of quantitation was 1 ng/mL and was determined as the lowest concentration within the linear portion of the calibration curves that had an accuracy within 15% of the nominal concentration. Accuracy and precision of the calibration curves were within 15%; the inter- and intra-day coefficient of variation was less than 5%.

**Pharmacokinetic evaluation**

The pharmacokinetic analysis was carried out by the Pharmacology Core Laboratory at the Colorado State University Flint Animal Cancer Center (Wittenburg). Non-compartmental pharmacokinetic analysis was carried out on the plasma CBD concentration-time data in all dosing groups using Phoenix WinNonlin Version 6.4 (Pharsight, Mountain View, California, USA) to obtain and compare pharmacokinetic parameters, determine dose proportionality, and predict pharmacokinetic parameters at different dose levels in each of the dosing formulations using nonparametric superposition. Parameters analyzed included maximal concentration ($C_{\text{max}}$), area under the curve (AUC), half-life ($T_{1/2}$), and dose-normalized values for $C_{\text{max}}$ and AUC.

**Statistical analysis**

All the outcome data on clinical significance were binary data. Contingency tables were constructed for each of the analyses and a Fisher’s exact test was conducted to evaluate significance between the formulations within each time point, as well as between the time points within each formulation for both doses of CBD. For some situations, in which all the subjects were in 1 group, no statistics could be done. For comparisons of the continuous data, such as the normalized peak area and CBD concentration between the formulation groups, a linear regression analysis was used, taking into account repeated measures across time points. The treatment effect was evaluated using the regression estimates and 95% confidence interval (CI). A $P$-value of 0.05 was considered to evaluate statistical significance for all analyses. SAS Version 9.4 (SAS Institute, Cary, North Carolina, USA) was used to analyze the data.

## Results

**CBD dosing formulations**

Calculated concentrations of CBD in the various formulations were 142.0 mg/mL in the 150 mg/mL CBD-infused oil, 77.6 mg/mL in the 75 mg/mL CBD-infused oil, 103.0 mg/mL in the 110 mg/mL CBD-infused transdermal cream, and 36.0 mg/capsule in the 50-mg capsule and 17.2 mg/capsule in the 25-mg capsule of the microencapsulated oil bead formulation. Although CBD was not equal to 100% of its labeled dose, the variability was < 10% for the CBD-infused oil and CBD-infused transdermal cream formulations (9.4% for the 150 mg/mL CBD-infused oil, 3.5% for the 75 mg/mL CBD-infused oil, and 6.4% for the CBD-infused transdermal cream). However, the amount of CBD per capsule varied considerably from the labeled amount (28% for the 50 mg/capsule and 31.2% for the 25 mg/capsule).

**Pharmacokinetic results**

Of the 30 dogs enrolled in the study, all dogs successfully completed the study. Plasma CBD concentrations were determined at 8 time points over the first 12 h after the initial dose of each formulation (Figure 1). The elimination half-lives of CBD-infused oil, microencapsulated beads, and infused transdermal cream formulations given as a single dose of 75 mg and 150 mg are listed in Table II.

Blood was collected for CBD plasma concentrations at 2, 4, and 6 wk. Median maximum plasma CBD concentrations (ng/mL) were higher for dogs receiving the CBD-infused oil formulation. The median $C_{\text{max}}$ and standard deviation for each group at 75 mg q12h and 150 mg q12h were as follows: CBD-infused transdermal cream $30.10 \pm 127.18$ and 97.46 + 476.10 ng/mL; microencapsulated oil beads $364.93 + 158.715$ and 546.06 + 287.14 ng/mL; and CBD-infused oil $649.43 + 164.34$ and 903.68 + 262.15 ng/mL, respectively. In addition, the overall exposure to CBD appeared to be dose-proportional in the CBD-infused oil formulation based on dose-normalized exposure values. The CBD-infused oil formulation appeared to have the smallest amount of inter-individual variability in plasma CBD exposure, as well as providing equal or greater plasma CBD exposures than the other 2 routes at each of the later time points (Figure 2).

Inter-individual variability in exposures was assessed as the standard deviation divided by the mean of the dose normalized $C_{\text{max}}$ and AUC ($C_{\text{max}}$/D and AUC_{0-inf}/dose, respectively) of each formulation after the first dose at both dose levels. With respect to the dose-normalized $C_{\text{max}}$ values, in the 75 mg q12h cohort, the calculated inter-individual variability for the CBD-infused oil group was 26.4% versus 48.9% and 167% for the microencapsulated oil beads and CBD-infused transdermal cream, respectively. In the 150 mg q12h cohort, the calculated inter-individual variability in the CBD-infused oil group was 22.1% versus 47.0% and 178% for the microencapsulated oil beads and the CBD-infused transdermal cream groups, respectively. The same pattern held true for the inter-individual variability in dose-normalized AUC in which the calculated values for the CBD-infused oil group versus the microencapsulated oil beads (75 mg q12h dose) was 33.0% versus 43.4% (AUC_{0-inf} could not be calculated in CBD-infused transdermal cream group due to lack of elimination phase). The inter-individual variability in AUC for the 150 mg q12h groups was found to be 29.4% and 32.3% in the CBD-infused oil versus microencapsulated beads, respectively.

A linear regression analysis taking repeated measures into consideration was used to evaluate the plasma CBD concentrations for each formula and its respective dose at 2, 4, and 6 wk. As normality was not met, the data were converted into log for analysis. With the exception of 2 wk in the 75 mg q12h group and 4 and 6 wk in the 150 mg q12h
group, the plasma CBD levels were higher in the CBD-infused oil than in the other 2 formulations. No significant difference was detected at 4 and 6 wk in the 75 mg q12h group (P-values 0.078 and 0.066, respectively). However, the differences were significant (P-values < 0.05) at all remaining time points, doses, and formulations. At 6 wk in groups given 150 mg q12h of their respective formulation, there was no significant difference among the forms of medication.

**Discussion**

We describe the first pharmacokinetic study of oral and transdermal CBD in healthy dogs receiving a dose of either 75 mg q12h or 150 mg q12h. In this 2-part study, CBD pharmacokinetics were measured during the initial 12 h of a single dose administration. In the second part, all dogs continued receiving their respective doses and delivery methods for a total of 6 wk, during which time, plasma CBD levels were maintained until the study’s completion.

Bioavailability of CBD has been reported to be low when given orally to both dogs and humans, presumably due to high first-pass effect through the liver (14,15). Our hypothesis was that a transdermal route of administration would avoid first-pass effect from the liver. Although bioavailability could not be determined in this cohort of dogs, we demonstrated that the CBD-infused transdermal cream did not reach similar plasma concentrations as the other 2 formulations. In general, transdermal absorption may be incomplete because of diffusion barriers, such as thickness of the skin of the pinnae or absorptivity of the CBD-infused transdermal cream. Since CBD is highly lipophilic, it accumulates within the stratum corneum of human and rodent skin and does not penetrate deeper skin layers (16,17).

Pharmacokinetic analysis demonstrated that the CBD-infused oil formulation resulted in higher maximal concentrations (Cmax) and systemic exposure (area under the curve; AUC) than the other 2 formulations (Table II). The oil formulation had the smallest amount of inter-individual variability in plasma CBD concentrations. This may be due, at least in part, to having less variation in the formulation. Regardless of cause, lower measurable plasma levels of CBD were evident in the CBD-infused transdermal cream group than in the groups given either of the other 2 formulations (Table II and Figure 1).

The half-lives (T1/2) reported in our study were shorter than those found in a previous crossover study evaluating plasma data in 6 dogs after intravenous administration of either 45 or 90 mg, followed by 180 mg orally (15). Terminal T1/2 ranged from 7 to 9 h in that study, but this was measured over 24 h after intravenous administration of CBD; the T1/2 of the oral dose could not be determined due to low or undetectable plasma levels. In the present study, CBD was not given via an intravenous route and sampling was carried out for 12 h following oral and transdermal doses. This difference in sampling duration may explain the longer T1/2 reported in the previous study, as this parameter is calculated by the slope of the terminal elimination phase, which tends to be more accurately represented the longer sampling can occur after drug administration.

Another limitation of this study is the short duration (6 wk) of CBD administration. Ideally, the dogs would be administered CBD for a longer period (several months at least) in order to assess whether the CBD concentrations remain stable, decrease, or increase over time.

Although we have demonstrated that CBD is absorbed orally, clinical trials are required to investigate its safety profile, to study...
its effectiveness in treating specific diseases, and to establish doses that provide therapeutic effects. Preferably, these studies would be conducted as prospective, double-blinded, placebo-controlled clinical trials.

Acknowledgments

The authors thank Jessica Prenni, Lisa Wolfe, Hend Ibrahim, and Crystal Badger at the Proteomics and Metabolomics Laboratory, Colorado State University for their technical assistance in developing the CBD assay. This study was funded in full by Applied Basic Science Corporation, which is the manufacturer of the medication used. The sponsor was not involved in study design, analysis or storage of data, or manuscript preparation. Dr. Stephanie McGrath was a < 5% shareholder in Applied Basic Science Corporation for the duration of the study. As principal investigator, Dr. McGrath’s duties included developing the study design, conducting the study, and participating in data analysis and manuscript preparation.

References

Does antimicrobial therapy improve outcomes in horses with severe equine asthma and a positive tracheal wash bacterial culture?

Michelle L. Husulak, Stephen T. Manning, Melissa D. Meachem, Hilary J. Burgess, Tasha Y. Epp, Julia B. Montgomery

Abstract

The objective of this study was to observe the outcomes of adding an antimicrobial treatment to a conventional treatment regime in horses with severe equine asthma in a clinical setting. Eleven client-owned horses with a history consistent with severe equine asthma, increased respiratory effort and nostril flaring, ≥ 20% neutrophils on bronchoalveolar lavage (BAL), and a positive tracheal wash (TW) bacterial culture were treated with environmental management, corticosteroids, and bronchodilators. Six horses were also treated with an antimicrobial (principal group), while the other 5 were administered saline as a placebo (control group). Treatment with antimicrobials significantly improved the post-treatment clinical score of the principal group compared with the pre-treatment score, whereas no significant difference occurred in the control group. The principal group also had significantly less neutrophil myeloperoxidase (MPO) activity post-treatment than pre-treatment, with a median difference of −0.39 units/[protein] in the principal group and a median difference of −0.21 units/[protein] in the controls. There was no difference in MPO activity pre- versus post-treatment in the control group. No differences were noted in the intra-group comparisons of pre- versus post-treatment BAL neutrophil counts, mucus scores, and concentrations of interleukin-8 (IL-8) or tumor necrosis factor-alpha (TNF-α) in bronchoalveolar lavage fluid (BALF) in either group. There were no differences found in the inter-group comparisons of the principal versus controls for each of the pre- and post-treatment time periods for BAL neutrophil count, mucus score, clinical scores, MPO activity, and IL-8 or TNF-α concentrations. The role of airway bacteria in horses with severe equine asthma requires further investigation as antimicrobial therapy improved post-treatment clinical scores and decreased MPO activity in the group of horses studied, but did not affect other measures of airway inflammation.

Résumé

L’objectif de la présente étude était d’observer dans un contexte clinique les résultats de l’ajout d’un traitement antimicrobien au traitement conventionnel de chevaux souffrant d’asthme sévère. Onze chevaux appartenant à des propriétaires et ayant une histoire correspondant avec de l’asthme sévère, un effort inspiratoire augmenté et un élargissement des narines, ≥ 20 % de neutrophiles dans le lavage broncho-alvéolaire (LBA), et une culture bactérienne positive à partir du lavage trachéal (LT) ont été traités par gestion de leur environnement, des corticostéroïdes, et des broncho-dilatateurs. Six chevaux ont également été traités avec un antimicrobien (groupe principal) alors que les cinq autres chevaux ont reçu de la saline à titre de placebo (groupe témoin). Le traitement avec les antimicrobiens améliorait de manière significative le score clinique post-traitement du groupe principal comparativement au score pré-traitement, alors qu’aucune différence significative ne fut notée dans le groupe témoin. Dans le groupe principal on nota également qu’il y avait significativement moins d’activité myéloperoxydase (MPO) des neutrophiles post-traitement comparativement à pré-traitement, avec une différence médiane de −0,39 unités/[protéine] dans le groupe principal et une différence médiane de −0,21 unités/[protéine] dans le groupe témoin. Il n’y avait pas de différence de l’activité MPO pré- versus post-traitement dans le groupe témoin. Aucune différence ne fut notée dans les comparaisons intra-groupe pré- versus post-traitement du dénombrement de neutrophiles dans les LAB, du score de mucus, et des concentrations d’interleukine-8 (IL-8) ou du facteur-alpha nécrosant des tumeurs (TNF-α) dans les liquides de lavage broncho-alvéolaire (LLBA) d’un groupe ou l’autre. Aucune différence ne fut trouvée dans les comparaisons inter-groupes du principal versus les témoins pour chacune des périodes de temps pré- et post-traitement pour le dénombrement des neutrophiles des LAB, le score de mucus, les scores cliniques, l’activité MPO, et les concentrations d’IL-8 ou de TNF-α. Le rôle des bactéries dans les voies respiratoires des chevaux souffrant d’asthme sévère nécessite des études supplémentaires étant donné que les thérapies antimicrobiennes ont améliorés les scores cliniques post-traitement et ont diminué l’activité MPO dans le groupe de chevaux étudiés, mais n’affecta pas d’autres mesures de l’inflammation des voies respiratoires.

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

Severe equine asthma, formerly known as recurrent airway obstruction (RAO) or heaves, is a chronic inflammatory condition of the lower airways of horses (1,2). It is characterized by neutrophilic inflammation of the lower airways in both acute exacerbations and chronic stages of the disease (3,4). Even though less well-characterized, equine asthma has some similarities to human asthma (5), specifically a subset of asthmatics who have predominantly neutrophilic airway inflammation (6). This has been correlated in humans with high airway bacterial counts (7) and it is now thought that the airway microbial community of asthmatics may be different than that of healthy humans (8).

The presence of bacteria in the airways of racehorses has been associated with inflammatory airway disease (IAD), now referred to as mild equine asthma, but their role in the pathogenesis of airway inflammation is not well-understood (9). Previously, bacteria within the airways of horses with chronic inflammation such as severe equine asthma have been considered a secondary problem associated with decreased airway clearance (10), even though scientific evidence to support this assumption is sparse. A positive tracheal wash (TW) bacterial culture in an asthma-affected horse creates treatment challenges for the attending veterinarian. Since the conventional treatment method for severe equine asthma includes systemic corticosteroids (11), there is concern for an infectious process occurring in the face of steroid-associated immunosuppression. Equine clinicians also need to ensure prudent use of antimicrobials to avoid side effects, such as diarrhea and antimicrobial resistance (12).

The purpose of this study was to determine if horses with severe equine asthma and a positive TW bacterial culture show improved clinical outcomes when antimicrobials are added to a treatment regimen of systemic corticosteroids, bronchodilators, and reduction of environmental allergens, compared to horses treated without antimicrobials. Our hypothesis was that horses treated with antimicrobials would have an improved clinical score and decreased markers of airway inflammation, including bronchoalveolar lavage (BAL) neutrophil count, BAL neutrophil myeloperoxidase (MPO) activity, and BAL tumor necrosis factor-alpha (TNF-α) and interleukin 8 (IL-8) concentrations, compared to horses receiving conventional treatment.

**Materials and methods**

**Horses**

Client-owned animals with a history and clinical signs consistent with severe equine asthma (exercise intolerance, mucopurulent nasal discharge, cough, respiratory distress) were included in the study. The owners of all horses signed a written consent form. The study was approved by the University of Saskatchewan Animal Care and Use Committee. Most samples were collected over a 2-year period between May and September, which is when most cases are seen in the practice area.

**Inclusion criteria**

Horse history included any of the following: multiple episodes of respiratory difficulty, including coughing, exercise intolerance, increased respiratory rate, increased respiratory effort, or nasal discharge. The horses had not been treated with antimicrobials, steroids, or bronchodilators within the 2 mo before inclusion in the study. On physical examination, all horses had an increased abdominal respiratory effort and/or nostril flaring at rest. The BAL cytological differential cell count had ≥ 20% neutrophils (13), the TW culture had growth of at least 1 bacterial species, and all species present were sensitive to the antimicrobial cefotiofur. No horses had evidence of systemic illness on physical examination or complete blood (cell) count (CBC) and there was no reported history or signs of laminitis. Horses were excluded if the primary caregiver declared an inability to comply with the treatment protocol.

**Owner questionnaire**

Before the examination, the horse owner or primary caregiver was asked a series of questions regarding clinical signs and management of the horses. A followup questionnaire regarding changes in clinical signs and management practices was completed at the second sampling date.

**Initial examination**

A distant examination was carried out and a clinical score with a 1 to 4 scale was assigned for abdominal effort and for nostril flaring at rest, respectively (14). The scores were then combined, with an overall score of 2 being normal and 8 being severe respiratory distress. The same clinician assigned the clinical score for both the pre- and post-treatment examinations on all of the horses. This clinician was blinded to assignment of horses to either the principal or control group. A full physical examination was carried out, including thoracic auscultation with rebreathing examination.

**Sample collection**

An upper airway endoscopy was conducted under standing sedation [detomidine (Dormosedan; Zoetis, Kirkland, Quebec), 0.01 mg/kg body weight (BW), intravenously (IV), and butorphanol (Torbugesic; Zoetis), 0.01 mg/kg BW, IV] using a 1.3-meter video endoscope. The amount of mucus within the trachea was scored on a 0 to 5 scale ranging from no mucus to profuse amounts (15). A TW was done by instilling 20 mL of sterile saline through a double-sheathed sampling catheter (Mila International, Florence, Kentucky, USA) into the trachea and aspirating back. Samples from the TW were placed in standard ethylenediamine tetra-acetic acid (EDTA) and plain serum tubes. Bronchoalveolar lavage (BAL) was carried out using a blind technique with a BAL catheter (Raftor 8 Products, Calgary, Alberta). Lidocaine (Lidocaine Neat; Zoetis) 20 mL, was instilled through the catheter to reduce coughing. Three aliquots of 120 mL of sterile saline were sequentially instilled and aspirated through the wedge BAL catheter. The first aliquot was discarded (4) and the second and third were pooled in a plain sample collection cup. Blood for CBC was collected from the jugular vein using an EDTA vacutainer tube. The CBC results were used to look for signs of systemic inflammation and were not used for further analysis. All samples were immediately placed on ice after collection and processed within 4 h in a commercial veterinary diagnostic laboratory (Prairie Diagnostic Services), except for a portion of the BAL fluid that was divided into 1-mL aliquots of the supernatant and frozen at −80°C along with the cell pellet.
All procedures were repeated at the end of the treatment protocol, 16 d after treatment was initiated.

Treatment protocols

All horses remained on their home farm and were treated by their primary caregiver. While the environment of each horse differed, all horses had a change to their management in order to reduce the volume of environmental allergens. This included at least 1 of the following: change in housing from indoors to outdoors or from a dirt paddock to pasture; change in feed to square bales, soaked hay, hay cubes, or pelleted feed; or withholding exercise in dusty arenas. All horses received a tapering dose of dexamethasone (Dexacort Powder; Rafter 8 Products) over 16 d (0.08 mg/kg BW, PO, q12h for 7 d).

Horses were randomly assigned to 2 groups using a random number generator in Excel (Microsoft Office 2007; Microsoft, Mississauga, Ontario) with 6 horses in the principal group and 6 controls. The principal group was treated with 2 doses of ceftiofur crystalline-free acid (Excede 200 Sterile Suspension; Zoetis) at 6.6 mg/kg BW intramuscularly (IM), 4 d apart, to provide 10 d of systemic antimicrobial coverage (16). The control group was treated with 0.9% sterile saline solution composed of 3 mM tetramethylbenzidine (Sigma-Aldrich, Oakville, Ontario) at 0.8 µg/kg BW, PO, q48h for 8 d) and received clenbuterol hydrochloride (Ventipulmin Powder; Rafter 8 Products) over 16 d (0.08 mg/kg BW, PO, q24h for 4 d, then 0.04 mg/kg BW, PO, q48h for 4 d, then 0.04 mg/kg BW, PO, q48h for 8 d) and received clenbuterol hydrochloride (Ventipulmin Syrup; Boehringer Ingelheim, Burlington, Ontario) at 0.8 µg/kg BW, PO, q12h for 7 d.

Horses were randomly assigned to 2 groups using a random number generator in Excel (Microsoft Office 2007; Microsoft, Mississauga, Ontario) with 6 horses in the principal group and 6 controls. The principal group was treated with 2 doses of ceftriaxone crystalline-free acid (Excede 200 Sterile Suspension; Zoetis) at 6.6 mg/kg BW intramuscularly (IM), 4 d apart, to provide 10 d of systemic antimicrobial coverage (16). The control group was treated with 0.9% sterile saline at an equivalent volume to the ceftriaxone and at the same time points. These treatments were administered by a veterinarian not otherwise involved with examining horses in the study. Both the veterinarian who examined the horses and provided the clinical scores and the horse owners were blinded to the groups.

Cytological analysis

The appearance of the TW and BAL fluid was assessed and direct and cytocentrifuge preparations (Cytospin 2; Shandon Southern Instruments, Sewickley, Pennsylvania, USA) were prepared and stained with a Romanowsky stain (Hema 3 Stain Set; Fisher Scientific, Middletown, Virginia, USA). Slides were examined by 1 of 2 Board-certified clinical pathologists who were blinded to the treatment protocol. A 200-cell differential count was conducted on the cytocentrifuge preparation on 100× magnification with immersion oil, as is standard protocol for the diagnostic lab used (Prairie Diagnostic Services). Slides were also assessed for cellularity, cellular preservation, mucus content, degree of cellular entrapment, the presence of respiratory epithelium, Curschmann’s spirals, environmental contaminants, and intra- and extracellular bacteria. The TW cytology results were used to rule out septic pneumonia by assessing neutrophil degeneration, as well as presence or absence of intracellular bacteria, and were not used for further analysis.

Bacterial culture

Tracheal wash (TW) fluid was cultured for bacterial identification and quantification using a quadrant streaking method (17) on agar plates. Samples were initially examined with Gram stain, then tested in aerobic and anaerobic conditions on Blood and MacConkey agar plates. Plates were incubated at 37°C. Results were considered positive if ≥ 5 homologous bacterial colonies were present in the first quadrant. Culture plates with < 5 colonies were considered negative as those colonies may represent sample contamination. Antimicrobial sensitivity was tested by the Kirby-Bauer Disc Diffusion method (18).

Neutrophil myeloperoxidase activity assay

An in-house assay, previously validated by our laboratory, was carried out to determine MPO activity. The bronchoalveolar lavage fluid (BALF) cell pellets from each sample were homogenized in 0.5 mL of 0.5% cetyltrimethylammonium chloride solution (Sigma-Aldrich, Oakville, Ontario) and 75 µL of sample or standard was placed in each well in a 96-well microplate in duplicates. Substrate solution composed of 3 mM tetramethylbenzidine (Sigma-Aldrich Canada), 120 µM resorcinol (Sigma-Aldrich), 2.2 mM hydrogen peroxide, and 2.2 mM potassium acetate was added to each well and were incubated for 30 min at room temperature. The reaction was stopped by the addition of 0.5 mL of 3 N sulfuric acid. The absorbance was measured at 450 nm. The absorbance of the sample was compared with a standard curve made with known concentrations of MPO.
peroxide (H₂O₂), and distilled water was added to the wells in equal parts. After 2 min, 150 μL of stop solution containing 1 M sulfuric acid (H₂SO₄) was added to each well. A microplate optical density reader was used to measure absorbance at 450 nm immediately after stopping the assay. The value obtained was expressed as MPO activity per protein concentration of each sample.

### Cytokine concentrations in BAL fluid

Bronchoalveolar lavage fluid (BALF) supernatant was thawed in 1-mL aliquots for use in commercially available enzyme-linked immunosorbent assays (ELISA) for TNF-α [DuoSet ELISA Equine TNF-α (R&D Systems, Minneapolis, Minnesota, USA)] and IL-8 (Horse IL-8 ELISA Kit; Cusabio, College Park, Maryland, USA). Assays were done according to manufacturers’ instructions and were repeated at least twice in quadruplicate. The quantification range for TNF-α was 3.6 to 500 pg/mL and for IL-8 was 3.9 to 500 pg/mL. Briefly, for the TNF-α ELISA, blank 96-well microplates were coated with equine TNF-α capture antibody, and samples or standard were added, followed by equine TNF-α detection antibody. Streptavidin-HRP was then added to each well, followed by a substrate solution and a stop solution. The optical density was determined with a microplate reader set to 450 nm. For the IL-8 ELISA, sample and standards were added to previously prepared 96-well microplates. Biotin-antibody was added to the wells, followed by HRP-avidin, TMB substrate, and then stop solution. The optical density was similarly determined.

### Data analysis

Data were analyzed with a commercial software program (Prism 6; GraphPad Software, La Jolla, California, USA). Nonparametric tests were used for not normally distributed data. Inter-group comparisons, between principal and control groups, were made at both pre- and post-treatment time points with a Mann-Whitney U-test to assess outcomes and ensure that there was no difference between the groups before treatment. Intra-group pre- and post-treatment values were compared with a Wilcoxon signed rank test. A P-value < 0.05 was considered significant.

### Results

Forty-five horses were examined, 33 of which had an abnormal clinical examination score (> 2) and therefore underwent sample collection. Nineteen horses (57.6%) had BAL neutrophils ≥ 20% and 24 horses (72.7%) had at least 1 bacterial species present on the TW bacterial culture. Only 11 horses had both a positive TW bacterial culture and ≥ 20% BAL fluid neutrophils and were therefore included in the study. These horses were a variety of breeds and consisted of 7 geldings, 3 mares, and 1 stallion, all between 8 and 23 y of age, with a mean age of 15 y. There was no difference in age between the groups. There were 6 horses in the principal group and 5 horses in the control. None of the horses had any signs of sepsis or overwhelming systemic inflammation on the CBC either pre- or post-treatment.

When inter-group comparisons were made between the principal and control groups at both the pre- and post-treatment time points, no significant differences were found for clinical score, mucus score, and percentage of BAL neutrophils, BAL neutrophil MPO activity, and concentrations of TNF-α or IL-8 in BAL fluid. When intra-group comparisons were made within the principal group, there was a significant improvement in clinical score (Figure 1) and BAL neutrophil MPO activity (Figure 2), from the pre-treatment to the post-treatment time points, with a P-value of 0.03 for both. There was no significant improvement of clinical score or BAL neutrophil MPO activity for the intra-group comparisons for the controls. Furthermore, there was no difference when comparing the change in difference of clinical score between the control and principal group (P = 0.39). The BAL neutrophil MPO activity had a median difference of −0.39 units/[protein] in the principal group and a median difference of −0.21 units/[protein] in the controls. No significant difference was noted in the intra-group comparisons.
between pre- and post-treatment values in either the principal or control groups for percentage of BAL neutrophils (Figure 3), mucus score (Figure 4), and concentrations of BAL fluid TNF-α (Figure 5) or BAL fluid IL-8 (Figure 6). No significant differences were found when the intra-group differences of pre- and post-treatment values were compared for either the principals or controls.

The bacteria cultured from the TW samples were highly variable in both groups, both pre- and post-treatment (Table I). All bacteria were sensitive to ceftiofur pre- and post-treatment except in 1 horse in the principal group, post-treatment, which grew ceftiofur-resistant *Actinobacillus* among other ceftiofur-sensitive bacterial species.

None of the 11 horses worsened throughout the course of treatment or developed any appreciable side effects.

**Discussion**

This study investigated the clinical and cytological response of horses with severe equine asthma to treatment with an antimicrobial along with conventional treatment, including environmental reduction of allergens, systemic corticosteroid, and bronchodilator treatment. The clinical score of horses treated with antimicrobials improved significantly between pre- and post-treatment intervals. While this intra-group improvement was not seen in the controls, the groups were small and the control group had a single outlier whose clinical score did not change after treatment. Furthermore, the change in the difference in clinical score between control and principal group did not vary, which highlights the need for further research in this area. There was no significant difference in the clinical scores between the groups before or after treatment.

Bronchoalveolar lavage (BAL) neutrophil counts were not different after treatment in either group, which is consistent with findings from studies where the environment was not optimal, i.e., ongoing exposure to environmental allergens, during treatment with dexamethasone (19). While it has been shown that airway reactivity does not always correlate with airway neutrophilia (20), studies in which horses were housed in an enclosed environmental chamber to control for environmental allergens have found a significant decrease in airway neutrophilia after inhaled steroid treatment (21). As this was a clinical trial using horses housed at home, environmental allergens were not completely eliminated, which may be responsible for the continued airway neutrophilia. The horse owners did everything that was economically and logistically possible to alter the management of horses enrolled in the study, but no 2 management systems are identical and a farm environment cannot replicate a research facility.

Expression of cytokines such as TNF-α can be up-regulated in BAL fluid from horses with severe equine asthma, which has been shown to decrease after treatment (21). In our study, there was no significant difference in cytokine concentrations in either the principal or control groups. Ideally, both messenger ribonucleic acid (mRNA) expression and protein concentrations would be evaluated to determine if protein translation is occurring. A significant decrease in IL-8 concentration pre- and post-treatment with inhaled fluticasone has been previously reported (21). The lack of significant change in concentrations of IL-8 and TNF-α in this study may be related to the environment the horses were housed in. Alternatively, the different results between studies may reflect a difference in cytokine profiles between experimentally induced acute exacerbations versus chronic clinical cases. Myeloperoxidase (MPO) activity was significantly improved in the principal group after treatment compared to pre-treatment values. It has previously been found that MPO concentrations are significantly elevated in BAL samples from horses in a severe asthma crisis (22). Our results are consistent with those findings and may suggest reduced airway neutrophil activation after antimicrobial treatment.

Ceftiofur crystalline-free acid was chosen for this study as it is labeled for the treatment of lower airway infections caused by *Streptococcus equi* ssp. *Zoopneumonicus* in horses (23). Although there were no negative clinical side effects to the use of antimicrobials in this study, 1 horse in the principal group had ceftiofur-resistant *Actinobacillus* spp. on TW culture after treatment. Ceftiofur
Table I. Results of tracheal wash (TW) bacterial culture from each horse pre- and post-treatment.

<table>
<thead>
<tr>
<th>Horse*</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
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<tbody>
<tr>
<td>Principal group</td>
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</tr>
<tr>
<td>A</td>
<td>1+ Streptococcus</td>
<td>1+ Enterobacter</td>
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<tr>
<td></td>
<td>1+ Staphylococcus</td>
<td>1+ Pseudomonas aeruginosa</td>
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<tr>
<td></td>
<td>2+ Pasteurella</td>
<td>2+ Actinobacillus</td>
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<tr>
<td>B</td>
<td>2+ Streptomyces</td>
<td>3+ Streptococcus</td>
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<tr>
<td></td>
<td></td>
<td>2+ Staphylococcus</td>
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<td></td>
<td></td>
<td>1+ Actinobacillus (Ceftiofur-resistant)</td>
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<tr>
<td></td>
<td></td>
<td>1+ Enterobacter</td>
</tr>
<tr>
<td>C</td>
<td>1+ Streptococcus</td>
<td>2+ Streptococcus</td>
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<td></td>
<td>1+ Actinobacillus</td>
<td>3+ Pasteurella</td>
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<td></td>
<td>1+ Actinobacillus suis</td>
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<td>G</td>
<td>1+ Streptococcus</td>
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<td></td>
<td>1+ Pasteurella</td>
<td>1+ Enterobacter</td>
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<td>2+ Actinobacillus suis</td>
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<td>H</td>
<td>2+ Streptococcus</td>
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<td></td>
<td>1+ Actinobacillus suis</td>
<td>1+ Pasteurella</td>
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<td></td>
<td>1+ Enterobacter</td>
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<td>K</td>
<td>1+ Streptococcus</td>
<td>Negative (enrichment culture only)</td>
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<td></td>
<td>1+ Actinobacillus suis</td>
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<td></td>
<td>1+ Acinetobacter</td>
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<td>Control group</td>
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<td>D</td>
<td>1+ Streptococcus</td>
<td>Negative (enrichment culture only)</td>
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<td></td>
<td>2+ Actinobacillus</td>
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<td></td>
<td>1+ Actinobacillus equuli</td>
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<td>E</td>
<td>1+ Corynebacterium</td>
<td>1+ Streptococcus</td>
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<td></td>
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<td>1+ Enterobacter</td>
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<td>F</td>
<td>1+ Streptococcus</td>
<td>Negative (enrichment culture only)</td>
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<td>1+ Actinobacillus</td>
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<tr>
<td></td>
<td>1+ Acinetobacter</td>
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</tbody>
</table>

* Each horse is represented by a letter assigned by the sampling date.

1+ indicates ≥ 5 homogenous bacterial colonies in the first quadrant, but < 5 in the second quadrant.
2+ indicates ≥ 5 colonies in the second quadrant, but < 5 in the third quadrant.
3+ indicates ≥ 5 colonies in the third quadrant, but < 5 in the fourth quadrant.
Bacterial culture was carried out with a quadrant streaking method.
crystalline-free acid was a practical antimicrobial choice for this study as the slow release and long-term action made it ideal for administration by a veterinarian involved in the study, which allowed the owner to be blinded to the treatment. Ceftriaxone was not the best choice in terms of antimicrobial resistance, however, as it is considered to be a Critically Important Antimicrobial by the World Health Organization (24).

While ceftriaxone is considered to be a Critically Important Antimicrobial by the World Health Organization (24), treatment of children with bacterial infections is generally considered to be a Critically Important Antimicrobial by the World Health Organization (24). As antimicrobial resistance is a concern, ceftriaxone may be the best choice in terms of antimicrobial resistance, however, as it is considered to be a Critically Important Antimicrobial by the World Health Organization (24). While ceftriaxone is not allowed the owner to be blinded to the treatment. Ceftriaxone was not the first choice when less important antimicrobials are available and appropriate based on bacterial sensitivity patterns.

It has previously been assumed that bacteria do not play a primary role in the pathogenesis of equine asthma (26). A subset of young racehorses with mild equine asthma (IAD) has been associated with abnormal or increased airway bacterial growth (9,27). We do not know if these bacteria are part of the cause or secondary to the inflammation and reduced airway clearance. The bacteria cultured from the TW samples herein were highly variable between horses and groups and the species found were reflective of what is believed to be normal commensal bacteria (28). Traditional culture methods tend to select for plate-adapted organisms, which causes the results to be biased towards anticipated species of bacteria based on sample type and history of what is typically cultured (29). The TW bacteria cultured from horses treated with antimicrobials tended to have a higher bacterial load and a wider variety of bacteria than the horses that did not receive antimicrobials. The authors considered this to be an interesting finding, which was difficult to interpret. As the samples were taken 6 d after the antimicrobial treatment period had ended, this may represent an airway microflora recolonization response.

This study had several limitations. Low horse numbers allowed outlier data to have a strong statistical influence, such as the outlier found in the principal group for the tracheal mucus score, as seen in Figure 1, and the outlier in the control group for the clinical score, seen in Figure 1. Housing horses at home, where the owners administered treatments and managed the environment had the potential to create excessive variability between the horses, making it difficult to ensure that the antimicrobial treatment was truly the variable that improved the clinical score and BAL MPO activity in the principal group. Ideally, this project would be repeated in a research setting with a complete reduction of environmental allergens. It would also be valuable to investigate the effects of different types of antimicrobials on the clinical outcome of horses with severe equine asthma.

In summary, bacterial growth from tracheal wash (TW) samples of horses with severe equine asthma can create challenges for equine veterinarians when it comes to choosing a course of treatment. These bacteria may play a role in the pathophysiology of the disease as there is some evidence that treatment with antimicrobials improved clinical scores and BAL MPO activity in this group of horses. In this study, the hypothesis stated can only be accepted in part, as treatment with antimicrobials may have improved the clinical score and MPO activity in horses with equine asthma, but did not improve the other variables studied. Further research is needed to fully understand whether antimicrobials have a beneficial effect on horses with severe equine asthma and a positive tracheal wash bacterial culture, as well as the role airway bacteria play in this disease process in general.

Acknowledgments

The authors thank Drs. Katherine Robinson and Katharina Lohmann for suggestions about study design and data interpretation, Dr. Stacy Anderson for assistance with the MPO activity assay, and Drs. Manuel Chirino and Sheryl Gow for advice on antimicrobial use. Hayley Kosolofski, Jordan Steedman, Alison Williams, Lea Riddell, Scott Dos Santos, and Louisa Belgrave assisted with sample collection and processing. Boehringer Ingelheim provided the Ventipulmin Syrup.

References

Impact of a trap-neuter-return event on the size of free-roaming cat colonies around barns and stables in Quebec: A randomized controlled trial

Valérie Bissonnette, Bertrand Lussier, Béatrice Doizé, Julie Arsenault

Abstract

The objective of this study was to evaluate the impact of a trap-neuter-return (TNR) event on the size of free-roaming rural cat colonies in Quebec. This prospective randomized, controlled study included 18 cat colonies around barns and stables that were randomly assigned to either a TNR group (10 colonies of 7 to 27 cats; 14.3 cats on average) or a control group (8 colonies of 7 to 26 cats; 14.5 cats on average). The number of cats in each colony was calculated from the images obtained by camera-trapping at: baseline (T0), 7.5 mo (T7), and 12 mo (T12). At baseline, the TNR group was subjected to a TNR event. When taking into account adults only, a significant growth difference was observed in the number of cats between the TNR group and the control group at T7 (P = 0.03). When including kittens as well as adults, a trend towards a lower growth of the TNR group compared to the control group was noted at T7 (P = 0.06). There was no difference in the number of kittens between the 2 groups at T7 (P = 0.49) or at T12 (P = 0.36). There was a trend towards more emigration in the control group at T12 (P = 0.095). Isolated TNR events have a low and temporary impact on colony size in Quebec’s rural cat colonies.

Résumé

L’objectif de cette étude est d’évaluer l’impact d’une intervention TNR sur la taille des colonies de chats en milieu rural québécois. Cette étude randomisée contrôlée impliquant 18 colonies de chats ayant accès soit à une écurie ou à une ferme. Les colonies ont été aléatoirement attribuées au groupe TNR (10 colonies de 7 à 27 chats; 14,3 chats en moyenne) et au groupe Contrôle (8 colonies de 7 à 26 chats; 14,5 chats en moyenne). Le groupe TNR a participé à un projet TNR au début de l’étude (T0). Par capture photographique, le nombre de chats et de chatons a été calculé, à 3 temps : T0, 7,5 mois (T7) et 12 mois (T12). Une différence significative de croissance des colonies du groupe Contrôle par rapport à celle du groupe TNR est notée à T7 lorsque seulement les adultes sont comptés (P = 0,03). Une tendance vers une plus faible croissance du Groupe TNR par rapport à celle du Groupe Contrôle est observée à T7 (P = 0,06), lorsqu’on inclut tous les individus. Aucune différence n’est notée lors de la comparaison du nombre de chatons des deux groupes (T7 P = 0,49 et T12 P = 0,36). Un nombre plus élevé de disparitions tend à être observé dans le groupe Contrôle à T12 (P = 0,095). Une intervention TNR isolée a un impact faible et temporaire sur la taille des colonies de chats en milieu rural québécois.

(Traduit par les auteurs)

Introduction

Domestic cat (Felis silvestris catus) overpopulation is a recognized problem worldwide (1,2). It is raising concerns not only because of the ethical issue of leaving numerous cats in poor living conditions without basic medical support (3), but also because domestic cats are a potential reservoir of infectious agents and represent a public health risk to humans and other animals (4,5). Domestic cats have endangered the survival of some species of wild cats by mating with individuals and creating hybrids (6). In addition to many threats from humans, domestic cats also threaten wildlife populations of small mammals, birds, and reptiles as they are skilful predators (7–10).

Free-roaming cats are widespread in rural areas, especially around barns and stables, which are ideal sites for domestic cat colonies. As cats are useful at controlling vermin at such sites, they are often provided with food and water and have access to many hiding places. Consequently, barns and stables have high carrying capacities for domestic cat populations and population growth is not controlled by attrition.

Several types of programs have been implemented in an effort to reduce feline overpopulation, but none has proved more effective than the others (11). Some theoretical models support the superiority of lethal methods over non-lethal ones (12,13), although their findings have not been proven in vivo. It must be taken into account that lethal methods are extremely difficult to apply in populated areas,
since humans develop compassion and affection for the free-roaming cats (14,15). In many situations, lethal methods are therefore not an option and are simply rejected as cruel (16).

Non-lethal methods, such as trap-neuter-return (TNR) programs, are an appealing alternative. Such programs have many advantages, such as decreasing reproductive rate, while increasing the well-being of individuals in the colony (15,17,18) and reducing the nuisance caused by cats (19,20). By improving the overall health of the cats, decreasing migration to other colonies (11,21), and providing a one-time vaccination, TNR programs could also decrease public health risk. Although some doubt the validity of such assumptions (22), such programs are perceived quite positively by the general public, which facilitates their implementation. On the other hand, some concerns have been raised as to whether TNR programs have a significant impact on the size of feline colonies (3,12,23). Hence, TNR programs increase survival and might enhance the carrying capacity by reducing intraspecies aggressive behavior (24–26). These programs also seem to promote the interest of the caretakers of the colony, making them more prone to dispense care to the cats (11).

Nevertheless, TNR programs have been used to control cat populations worldwide for at least the last 30 y (27). Substantial financial and human resources are allocated to run those programs. To our knowledge, the impact of a TNR event on the size of colonies around barns and stables has never been evaluated. Furthermore, no research has been published on the efficiency of TNR programs in a temperate climate with harsh winters, similar to Quebec. The results of only 13 field trials addressing cat population control have been published (14,23,28–38).

The objective of this study was to evaluate the impact of a one-time TNR event on the size of free-roaming cat colonies around barns and stables, by using a randomized, controlled trial. We hypothesized that implementing an intensive TNR event would significantly decrease the number of free-roaming cats in colonies around barns and stables over a 1-year period.

Materials and methods

Colony selection

The study took place from May 2014 to August 2015. Invitations were sent to colony caretakers through the bovine and equine ambulatory services of our institution. The first 20 colonies that were sent to colony caretakers through the bovine and equine taken part in a TNR event. Colonies were excluded if they had previously been published (3,12,23). Hence, TNR programs increase survival and might enhance the carrying capacity by reducing intraspecies aggressive behavior (24–26). These programs also seem to promote the interest of the caretakers of the colony, making them more prone to dispense care to the cats (11).

Nevertheless, TNR programs have been used to control cat populations worldwide for at least the last 30 y (27). Substantial financial and human resources are allocated to run those programs. To our knowledge, the impact of a TNR event on the size of colonies around barns and stables has never been evaluated. Furthermore, no research has been published on the efficiency of TNR programs in a temperate climate with harsh winters, similar to Quebec. The results of only 13 field trials addressing cat population control have been published (14,23,28–38).

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Data collection

The size and composition of the colonies were measured at each time point by using an adapted method of camera-based observations that was described in a previous study (39). Numeric recorders with 2 camera heads were placed 1 to 2 m away from the main feeding or resting area and were left to collect data for 72 consecutive hours. Recorders were motion-activated. Images were then viewed and analyzed so that all individuals were identified. The principal investigator (VB) carried out all these steps to prevent observer bias. To validate the methodology, videos of 5 colonies at one time point were randomly selected to be reviewed and analyzed by a second observer for comparison purposes.

Colony size was defined as the number of individuals in each colony. The data collected included the number of adults, the number of kittens, and the total number of cats in each colony. Young individuals with no permanent canines, thus less than 6 mo old, were considered kittens and cats older than 6 mo were considered adults. The number of spayed and neutered individuals following the TNR protocol application in the TNR group and the number of individuals that remained on site for the duration of the study were also reported.

TNR procedure

An intensive trapping effort took place for 48 h in each colony from the TNR group immediately after the size of each colony had been evaluated by camera. Social individuals were caught by hand and placed in carriers, while semi-feral and feral cats were caught using trapping cages with several baits. All captured cats were brought to the Faculty of Veterinary Medicine at the University of Montreal (FVM-UofM) where the surgical procedures were carried out by veterinarians only. Students of FMV-UofM were invited to participate, but only under the direct supervision of a veterinarian. Kittens that were too young at the time of trapping were left on site if weaned or otherwise brought with the mother to FVM, but were left intact. Cats weighing more than 0.5 kg and that were more than 8 wk of age were subjected to a TNR protocol.

The study were randomly assigned to either the experimental group (referred to as the TNR group) or the control group. Data on the size and composition of each colony was collected by video camera at 3 time points: T0 (baseline), T7 (32 +/- 2 wk), and T12 (52 +/- 1 wk). Immediately after data were collected at T0, cats from the TNR group were subjected to a TNR protocol.

Statistical analyses

The comparability of the TNR and control group at randomization was evaluated by comparing the 2 groups at T0 using an exact bilateral Wilcoxon for 2 variables: the total number of cats and the number of adults in each colony at T0. Both groups were comparable with respect to these variables, as assessed by the Wilcoxon rank sum test.
number of adult cats. To evaluate the impact of the TNR event on colony growth, the difference in colony size between T0 and T7 (ΔT7–T0) and between T0 and T12 (ΔT12–T0) was calculated for the number of adult cats, number of kittens, and total number of cats, respectively. These differences were then compared between the TNR and the control group using an exact unilateral Wilcoxon test, testing the hypothesis that the TNR event will result in a lower growth in colony size over time. Furthermore, the number of adults observed at T0 that left their colony at T7 and T12 was also counted in both groups and then compared using the exact unilateral Wilcoxon test. We concluded that there was a statistically significant difference when P < 0.05. A trend was considered when P < 0.1.

**Results**

The TNR group included 10 colonies of 7 to 27 cats, consisting of an average of 14.3 cats/colony, with a median of 13.5 cats/colony and a total of 128 adults and 15 kittens. For the control group, 2 of the colonies had to be excluded due to data loss at T0. Therefore, the control group included 8 colonies of 7 to 26 cats, consisting of an average of 14.5 cats/colony, with a median of 12.5 cats/colony and a total of 116 adults and 23 kittens at T0. At T0, the size of colonies in the TNR group was similar to the control group (P = 0.78 for all cats or P = 0.95 for adults only, exact bilateral Wilcoxon test). The data obtained by the second observer was similar to the principal investigator’s observations.

Between 67% and 100% (median of 96%) of cats were put through TNR in each colony of the TNR group, for an average of 92% of cats per colony. Slightly more than half (53%) of the individuals sterilized were females. There was no perioperative death and only 1 minor wound infection in a female was reported. At T7, colonies in the TNR group consisted of 87% of spayed or neutered individuals on average (median of 90%). Similarly, at T12, colonies in the TNR group were composed of 87% sterilized individuals on average (median of 91%).

The median numbers of cats per colony at the different time points for each group are presented in Table 1. When taking into account adults only, a significant difference in colony growth was observed at T7 (i.e., ΔT7–T0) between the TNR group and the control group (P = 0.03). On median, there was an increase of 2.5 adult cats per colony at T7 compared to T0 for the control group, whereas the same number of adult cats at T7 and T0 was observed for the TNR group. The TNR event had no significant impact on the growth of the colonies when comparing all individuals (kittens and adults), but a trend was observed (P = 0.06). On median, there was 0.5 more cats at T7 compared to T0 for colonies in the control group, whereas a reduction of 2 cats was observed in the TNR group. There was no difference in the number of kittens in the TNR group compared to the control group at T7 versus T0 (P = 0.49). No difference in colony growth was observed at T12 versus T0 (i.e., ΔT12–T0) for adults (P = 0.25), for kittens (P = 0.36), or for all cats (P = 0.21).

The number of cats that left their colony between T0 and T7 was not significantly different between the 2 groups (P = 0.3) (Table 1). Nevertheless, there was a trend towards more disappearances in the control group than in the TNR group between T0 and T12 (median of 7.5 cats left colonies in the control group and a median of 3 cats left colonies in the TNR group, P = 0.095).

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<th>Table I. Median number of cats per colony for the control and TNR groups at different time points.</th>
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* Number of cats identified at T0 that were still seen later in the colonies.

T0 — baseline; T7 — 7.5 mo; T12 — 12 mo.

**Discussion**

We hypothesized that implementing a one-time intensive TNR event would significantly decrease the size of free-roaming cat colonies around barns and stables over a 1-year period. Our hypothesis was partially supported as we observed a significant decrease in the growth of the TNR group 7 mo after the application of the event when taking into account adults, but this difference was not significant at 12 mo or when considering all individuals (kittens and adults). The impact of our TNR intervention was therefore considered as low and temporary.

As this was the first project to study the impact of a TNR event on cat colonies around barns and stables over a 1-year period, it is not possible to compare it with other studies. A few studies in other contexts, however, have reported positive results of TNR programs in controlling cat populations. A study on the impact of a TNR program on rural cat colonies was conducted in North Carolina from 1998 to 2005 (28). Six cat colonies (consisting of an average of 14 individuals) were reduced by 36% after 2 y of participation in the program. All cats were sterilized and a vasectomy was carried out on the males of 3 of those 6 colonies instead of castration. The size of the colonies continuously declined over the years. By the end of the trial, 1 colony had dissolved, while the 5 others were reduced to 5 or less individuals (28). Unlike our project, trapping efforts were constant throughout the study and caretakers were relied on to bring in the cats progressively over the years. Use of vasectomy could also explain the success obtained since this procedure allows males to maintain their aggressive intraspecies behavior, their boldness, and their mating habits. In the case of mating habits, however, vasectomy was not proven more efficient than castration for population control (28).

One other study published results of their observations after the first year following the introduction of a TNR program. In 132 colonies from Florida, consisting of an average of 7 cats, a decrease of 27% was noted over a year (14). Our project resulted in a decrease of almost half (14% of decrease at T12) what this study obtained. Their project was not controlled, however, and the caretakers were responsible for the cats participating in a TNR program and for calculating the number of cats per colony.

Field trials have had encouraging results in controlling cat populations with TNR programs after multiple years of effort. A population of 155 cats on a university campus in Florida was decreased to 23 cats in 11 y of TNR efforts combined with an intensive adoption
program (29). Studies in Rome and Rio de Janeiro were conducted over multiple years and populations decreased by 22% and 58%, respectively (30,31).

The success of a TNR program at controlling population is thought to strongly relate to the number of individuals that are sterilized. Some theoretical models suggest that it is possible to control population with TNR programs, but only with high proportions of sterile individuals, i.e., 51% to 94% (11,12,28,32,40,41). This contradicts the results of this study since no significant decrease was observed even with a high sterilization rate (average of 92% at T0 and 87% at T7 and T12). It was quite simple to trap most of the cats, probably because the colonies were already well-established, the cats received food regularly from the caretaker most of the time, and most individuals were not completely feral, with some even socialized. Our results are in accordance with a study using a mathematical model, which suggested that in colonies where immigration is possible, i.e., an open system, application of high treatment rates of TNR would result in a slight decrease or no change in the population size after a year (11). Indeed, this project was carried out in an open system in which cats were free to leave or enter the colonies and researchers had no control over people abandoning their pets on the colony territory.

In this trial, the number of adults in the TNR group decreased significantly compared to the control group at T7 only. One possible explanation for this short-term success would be that fewer kittens in the TNR group grew into adults. This would have happened for 2 main reasons: first, the intervention took place in early summer, which interrupted some gestations (62 fetuses were aborted) and prevented others from happening in the second peak of reproduction in late summer and second, that some unweaned kittens were inevitably left alone at the colony while their mother was being spayed. Even if lactating females were released as soon as possible after surgery, the absence of the mothers for several consecutive hours might have jeopardized the kittens’ survival.

There was no difference in the growth of the TNR group compared to that of the control group after a year. The weather during the winter of 2014/2015 was the harshest in 20 y, with the coldest temperatures ever reported in Quebec in February. These harsh conditions could have affected the results of this study by having an impact on mortality and decreasing the reproductive rate of the control group. This would have made it harder to make a difference in population growth and the number of kittens.

Other factors that could partially explain the low impact of our intervention are frequency of implementation, failure to remove socialized individuals, and short follow-up period. Unlike the other studies discussed, our project involved only a one-time TNR event. Catching/trapping was carried out intensively over a 48-hour period. New members that integrated into the colonies during the year were not spayed or neutered. It would probably have been beneficial to continue the TNR effort throughout the year. Another option would have been to return 3 mo after the first trapping period to spay and neuter the kittens that were left intact at that time because of their young age. Removal of kittens and socialized adults with good potential for adoption would also have led to a faster decrease in population size, as indicated by the results of some successful TNR programs that joined their efforts with animal shelters (28,29,33,34,36–38,42). Finally, as the life expectancy of an adult in a free-range cat colony is less than 5 y (43), the population may have decreased in size over time due to age or accidents.

Another reason for the absence of statistically significant differences may be related to the low statistical power of a small sample size. Based on a posteriori analysis, the statistical power was estimated at 51% for the comparison of colony growth at T7 versus T0 between the 2 groups for the total number of cats. To reach a statistical power of 80% given the differences observed, 21 colonies would have been required in each group, for a total of 42 colonies. Limited resources and time contributed to restricting the sample size. Retrospectively, the authors consider it would have been unrealistic to double the size of the sample with the method of evaluation chosen, as the camera-trapping method was both time- and energy-consuming.

Several other interesting results were noted in our study. First, there was a trend towards more disappearances in the control group than in the TNR group between T0 and T12. These disappearances could have been due either to emigration or death. Female emigration is motivated by the search for a new source of food, while males leave in search of new mates. It therefore makes sense to observe the same trend in the control group colonies, which had better survival rates. The TNR group was also vaccinated and received a dose of anti-parasitic agent, which could have improved the health of cats in this group.

Second, domestic cats generally live alone, with groups of females occasionally gathering around a food source (21). This was not observed in the present study, since both males and females were forming colonies around barns and stables and living close to each other. Third, we reported smaller growth in population than what was suggested in previous reports (28,32). This was expected as most other studies took place in more welcoming climates. It is also possible that the colonies included in this study were already close to their carrying capacity.

Finally, an unexpectedly small number of kittens was identified in all colonies. While theoretically cats are very fecund (25), this could have been diminished by the harsh winter climate in Quebec. It is also possible that the method of data collection was less efficient at counting kittens since the cameras were static and located in the main feeding or resting areas. Unweaned kittens are generally hidden from other cats in the colony, as well as from predators and humans, and would not usually be seen in the main areas.

Inevitably, we identified potential bias in this study. A selection bias could be present, since only colonies with motivated caretakers who answered the invitation took part in the study. Most of these caretakers were providing a regular food and water supply to their cat colony and some were even providing basic medical care, such as wound management and ocular topical treatments, to
the social individuals and kittens. As a result, most colonies were well-established and consisted of relatively social individuals and few feral ones. This might have facilitated the trapping, which enabled us to sterilize most of the cats in the colonies. Despite this, these motivated caretakers were increasing the carrying capacity of their territory, which could have reduced the impact of population control interventions.

Finally, we could not assess the precision of the use of cameras as a method for measuring the colony size. Although this is an objective method, the colony size could be underestimated if some individuals did not visit the main site of activity in the colony, as is probably the case for young kittens. Moreover, it can sometimes be challenging to identify all individuals when colors and patterns of coats and silhouettes are similar. Finally, data can easily be lost when the intensity and direction of natural light changes, the cameras accidentally get displaced, or unsuccessful data transfers occasionally occur.

It was concluded that a one-time TNR event had a low and temporary impact on the size of cat colonies in this study. While these results do not discourage the use of TNR events in rural cat colonies, they strongly suggest that a one-time intensive TNR event might not be worth the effort if there is no possibility of a continuous trapping effort to counteract immigration.

This study is a first step towards a better understanding of the true impact of TNR events on cat colonies in rural environments in a temperate climate with harsh winters. Although many TNR programs are being carried out, their results are not being evaluated. More field studies should be conducted in different settings in order to gain a better understanding of the main factors influencing the impact of TNR events. Given the scarce resources available for cat population control, it is essential that efforts are deployed the most efficient way possible and this can only be achieved if we have a good understanding of the problem.

Acknowledgments

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References

Cardiac weights and weight ratios as indicators of cardiac lesions in pigs: A study of pig hearts from an Ontario abattoir

Kathy Zurbrigg, Tony van Dreumel, Max F. Rothschild, David Alves, Robert Friendship, Terri L. O’Sullivan

Abstract
Clinically healthy pigs used in research are assumed to have normal cardiac structure and function. Subclinical cardiac abnormalities may adversely affect the responses being measured in these experiments. The gross and histologic lesions observed in hearts collected from a Canadian abattoir between 2012 and 2015 indicated an unexpectedly high prevalence of cardiac abnormalities: 75% (297/396) of the hearts examined had such lesions. The ratios of total heart weight to body weight and of right ventricle weight to body weight were significantly greater for the hearts with lesions than for the hearts with no lesions, which suggests that cardiac remodeling, particularly hypertrophy, had occurred. The large percentage of hearts with cardiac remodeling from asymptomatic market pigs demonstrates an increased probability that subclinical cardiac abnormalities may exist in research pigs, especially those accessed through commercial channels. Researchers should be aware of this likelihood if subclinical cardiac abnormalities could adversely affect their experimental findings.

Résumé
Les porcs cliniquement en santé utilisés en recherche sont présumés avoir une structure et fonction cardiaques normales. Des anormalités cardiaques sous-cliniques peuvent affecter de manière adverse les réponses étant mesurées dans ces expériences. Les lésions macroscopiques et histologiques observées dans les cœurs amassés d’un abattoir canadien entre 2012 et 2015 ont indiqué une prévalence élevée inattendue d’anormalités cardiaques : 75 % (297/396) des cœurs examinés avaient de telles lésions. Les ratios poids total du cœur/poids corporel et poids du ventricule droit/poids du corps étaient significativement plus élevés pour les cœurs avec lésions comparativement aux cœurs sans lésions, ce qui suggère qu’un remodelage cardiaque, particulièrement une hypertrophie, est survenu. Le pourcentage élevé de cœurs avec remodelage cardiaque provenant de porcs asymptomatiques prêts pour le marché démontre une probabilité accrue que des anormalités cardiaques sous-cliniques peuvent exister chez des porcs utilisés en recherche, spécialement ceux obtenus via des voies commerciales. Les chercheurs devraient être au fait de cette possibilité si des anormalités cardiaques sous-cliniques pouvaient affecter négativement leurs trouvailles expérimentales.

Introduction
Pigs are frequently used as a model for human cardiac research because of the similar heart size and vasculature (1,2). The intrinsic responses of dilation and hypertrophy are also similar (2); therefore, pigs have been used to study the pathophysiological and gene-expression responses of syndromes such as atrial fibrillation (3,4). During times of increased stress or physical exertion, pigs may display symptoms of cardiac insufficiency and die suddenly in research and commercial environments (1,2,5,6). Investigations in swine-production settings have suggested that structural abnormalities such as hypertrophy may be responsible for a portion of on-farm and in-transit losses of pigs (7,8). Differences in the QT interval and P wave of the electrocardiograms of some pigs may also indicate susceptibility to prolonged ventricular repolarization, arrhythmias, and sudden death (1).

Domestication and intense genetic selection may have influenced not only carcass traits and growth performance but also, unintentionally, cardiac size and function (9). The ratio of heart weight to body weight in wild boars has been reported as 0.42% to 0.64% (9,10), whereas the same ratio is 0.3% for a modern sow (10) and 0.27% to 0.44% for a growing pig (10,11). Reduced cardiac size has been suggested to affect the pig’s adaptability in cardiac response to increased exertion, thus leading to a greater risk of cardiac insufficiency (8,9).

Criteria used in research to determine that a heart is normal are often not stated or are based on the absence of a specific abnormality of interest in the study. Undetected cardiac lesions or structural changes may affect the pathophysiological responses and gene expression recorded in experiments with pigs. The use of heart weight and the ratio of heart weight to body weight should allow the detection or confirmation of gross cardiac lesions in hogs that outwardly appear healthy. This study was conducted to detect the
presence of cardiac abnormalities in healthy pigs and to determine whether increased postmortem cardiac weight and the ratio of heart weight to body weight could predict the presence of gross cardiac lesions.

**Materials and methods**

Hearts were collected from the processing line of 1 of the 3 largest pig abattoirs in Ontario between June 2012 and April 2015. At the time of collection, the abattoir slaughtered 15,000 pigs aged 5 to 7 mo per week. On each of 37 d over the study period 10 to 15 hearts were selected from the processing line, by 2 methods. First, the 5th heart was picked from each unique group of carcass tattoos (farm identifiers) as they came down the line until 10 hearts had been collected from each of 10 unique tattoos, for a total of 100 hearts. Second, 30 unique tattoos were selected from a list of all tattoos representing farms shipping more than 1000 pigs to the abattoir in 2013 (the most recent full year of data at the time). These tattoos were selected randomly by starting half-way down the list and then selecting every 6th tattoo until 30 had been obtained. The abattoir contacted the selected owners to determine if they would participate in the study. If an owner did not want to participate, the next owner from the list was contacted. This process was repeated until 30 owners agreed to participate. The abattoir notified the researcher (K.Z.) of the date, time, and number of pigs to be shipped for each of those 30 owners, and she collected 10 hearts from each of the 30 tattoos by selecting from the line every nth heart, where n = the total number of pigs shipped from that tattoo that day divided by 10. Agreement by the owners to participate in the study was required because this group of hearts was to be used for another study.

Upon removal from the processing line the hearts were put in resealable freezer bags, placed in a cooler with ice packs, and transported to the Animal Health Laboratory at the University of Guelph, Guelph, Ontario. The hearts were placed intact into 10% formalin within 2 h of collection for future examination by a pathologist (T.V.D.) using the standardized protocol outlined below.

Owing to abattoir protocols, the pig’s body weight (BW) corresponding to each heart collected from the processing line was not available. Therefore, heart weight to body weight ratios were calculated by use of the lowest and the highest body weights for each shipment from a specific tattoo (data not included or shown). The average body weight of all pigs in that shipment was used as a proxy for the body weight of each pig in that shipment whose heart was collected from the processing line.

After a minimum of 7 d of fixation in formalin, visible clots were removed from the major vessels, noncardiac tissue was trimmed away, and the aorta and pulmonary artery were trimmed to their bases. The heart was weighed and the weight recorded as total heart weight (THW) in grams. If clots were found on opening of the heart they were removed and weighed; the weight of the clots was subtracted from the THW. The atria were removed from the ventricles. The right ventricle (RV) was removed from the left ventricle and septum (LV + S) by cutting along the coronary grooves. The RV and then the LV + S were weighed separately in grams and those weights recorded. The following weight ratios were calculated: LV + S/RV, THW/BW, LV + S/THW, RV/THW, LV + S/BW, and RV/BW.

Hearts were examined grossly for the presence of thickening and nodularity of all valves (endocardiosis), dilation and hypertrophy of the ventricles, dilation of the atria, and dilation of major vessels. Absence of a lesion was scored as 0 and presence as 1. Sections of the RV, anterior LV, posterior LV, and septum of each heart were examined histologically for the presence of medial hyperplasia and perivascular fibrosis of the intramural coronary arteries and for interstitial fibrosis. Absence of these lesions was scored as 0 and presence as 1.

After the heart weights and the data on gross and histologic lesions were recorded, the hearts were classified as “lesion-free” (no gross lesions and a score of 0 for histologic lesions), having “no gross lesions” (no gross lesions but a score of 1 for histologic lesions), or having “lesions” (a score of 1 for at least 1 of the gross and histologic lesions listed).

The statistical program Stata (Stata Statistical software, Version 14; Stata Corporation, College Station, Texas, USA) was used to calculate the mean and standard deviation (SD) of the heart weights, body weights, and weight ratios. A 1-way analysis of variance (ANOVA) with a Bonferroni test for multiple comparisons was used to determine if these measures were statistically different between the groups of hearts.

**Results**

To be paid the greatest price for their hogs, producers must be within a weight and back-fat range specified by their abattoir. This results in low variation of within-load body weight and is the reason the use of the lowest or highest body weight of pigs in a single shipment from a specific tattoo resulted in the same trends and significant variables as when the average body weight of the pigs in that shipment was used.

Although 400 hearts were selected from the processing line and examined, only 396 had a complete dataset for heart weights and body weight and are represented in the results. Only 6% of the hearts (23/396) were free of both gross and histologic lesions, whereas 19% (76/396) were free of gross lesions but had histologic lesions, and 75% (297/396) had both types of lesions. Table I lists the mean and SD of the heart weights, body weight, and weight ratios of the 3 categories of heart together with the ANOVA results. There was a significant difference \(P < 0.05\) between all heart categories in the LV + S/RV ratio, the greatest value being for “lesion-free” hearts, followed by “no gross lesions,” and then “lesions.” Body weight was significantly greater \(P < 0.05\) in the category of “lesions” compared with the other 2 categories. The ratios THW/BW, LV + S/BW, and RV/BW were all significantly greater \(P < 0.05\) for the category “lesions” compared with the category “no gross lesions,” and the ratios THW/BW and RV/BW were significantly greater \(P < 0.05\) for the “lesions” category compared with the category “lesion-free.” There were no significant differences in heart weight ratios between the categories “no gross lesions” and “lesion-free.”

**Discussion**

The selected hearts had passed a visual inspection by plant inspectors for obvious defects or disease (e.g., fibrosing pericarditis), and
Table 1. Comparison of weights and weight ratios in 3 categories of hearts of commercial market hogs collected from the processing line of an Ontario swine abattoir.

<table>
<thead>
<tr>
<th>Variable&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lesions (n = 297)</th>
<th>No gross lesions (n = 76)</th>
<th>Lesion-free (n = 23)</th>
<th>Results of analysis of variance&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean&lt;sup&gt;b&lt;/sup&gt; (standard deviation)</td>
<td></td>
<td></td>
<td>SS</td>
</tr>
<tr>
<td>THW (g)</td>
<td>411.75&lt;sup&gt;d&lt;/sup&gt; (49.42)</td>
<td>378.36&lt;sup&gt;de&lt;/sup&gt; (38.06)</td>
<td>370.41&lt;sup&gt;d&lt;/sup&gt; (45.83)</td>
<td>89 105.02</td>
</tr>
<tr>
<td></td>
<td>879 922.20</td>
<td>394</td>
<td>2233.30</td>
<td></td>
</tr>
<tr>
<td>LV + S (g)</td>
<td>254.61&lt;sup&gt;d&lt;/sup&gt; (29.61)</td>
<td>240.29&lt;sup&gt;de&lt;/sup&gt; (23.42)</td>
<td>243.06&lt;sup&gt;d,e&lt;/sup&gt; (28.12)</td>
<td>13 069.09</td>
</tr>
<tr>
<td></td>
<td>318 877.27</td>
<td>394</td>
<td>809.33</td>
<td></td>
</tr>
<tr>
<td>RV (g)</td>
<td>95.12&lt;sup&gt;d&lt;/sup&gt; (14.00)</td>
<td>85.49&lt;sup&gt;de&lt;/sup&gt; (11.16)</td>
<td>79.57&lt;sup&gt;e&lt;/sup&gt; (11.29)</td>
<td>9564.39</td>
</tr>
<tr>
<td></td>
<td>70 277.31</td>
<td>394</td>
<td>178.36</td>
<td></td>
</tr>
<tr>
<td>BW (kg)</td>
<td>124.82&lt;sup&gt;d&lt;/sup&gt; (5.53)</td>
<td>122.61&lt;sup&gt;de&lt;/sup&gt; (5.60)</td>
<td>119.98&lt;sup&gt;e&lt;/sup&gt; (5.11)</td>
<td>748.00</td>
</tr>
<tr>
<td></td>
<td>11 981.50</td>
<td>394</td>
<td>30.41</td>
<td></td>
</tr>
<tr>
<td>LV + S/RV</td>
<td>2.71&lt;sup&gt;d&lt;/sup&gt; (0.34)</td>
<td>2.83&lt;sup&gt;de&lt;/sup&gt; (0.29)</td>
<td>3.10&lt;sup&gt;d&lt;/sup&gt; (0.49)</td>
<td>4.43</td>
</tr>
<tr>
<td></td>
<td>46.63</td>
<td>394</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>THW/BW (g/kg)</td>
<td>3.30&lt;sup&gt;d&lt;/sup&gt; (0.38)</td>
<td>3.09&lt;sup&gt;de&lt;/sup&gt; (0.31)</td>
<td>3.09&lt;sup&gt;e&lt;/sup&gt; (0.35)</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>53.81</td>
<td>394</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>LV + S/THW</td>
<td>0.62&lt;sup&gt;d&lt;/sup&gt; (0.04)</td>
<td>0.64&lt;sup&gt;de&lt;/sup&gt; (0.03)</td>
<td>0.66&lt;sup&gt;e&lt;/sup&gt; (0.04)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.54</td>
<td>394</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>RV/THW</td>
<td>0.23&lt;sup&gt;d&lt;/sup&gt; (0.02)</td>
<td>0.23&lt;sup&gt;de&lt;/sup&gt; (0.02)</td>
<td>0.21&lt;sup&gt;e&lt;/sup&gt; (0.03)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.21</td>
<td>394</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>LV + S/BW (g/kg)</td>
<td>2.04&lt;sup&gt;d&lt;/sup&gt; (0.23)</td>
<td>1.96&lt;sup&gt;e&lt;/sup&gt; (0.21)</td>
<td>2.03&lt;sup&gt;d,e&lt;/sup&gt; (0.21)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>20.64</td>
<td>394</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>RV/BW (g/kg)</td>
<td>0.76&lt;sup&gt;d&lt;/sup&gt; (0.11)</td>
<td>0.70&lt;sup&gt;e&lt;/sup&gt; (0.09)</td>
<td>0.67&lt;sup&gt;e&lt;/sup&gt; (0.09)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>4.48</td>
<td>394</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> THW — total heart weight; LV + S — weight of left ventricle plus septum; RV — weight of right ventricle; BW — body weight.

<sup>b</sup> Means sharing the same superscript (d,e,f) are not significantly different from each other (P < 0.05).

<sup>c</sup> For each variable with 2 results, the top result is for the between-group comparison and the bottom result for the within-group comparison.

SS — sum of squares; DF — degrees of freedom; MS — mean squares; F — calculated F ratio.

before their examination the researchers assumed that all selected hearts would be free of cardiac lesions. The gross and histologic lesions observed are associated with compensatory cardiac remodeling in pigs and humans (12–14). The lesions did not indicate a specific cause of the remodeling owing to the limited intrinsic responses and shared molecular pathways involved in cardiac compensation (15,16). However, remodeling may be due to cardiac unfitness in the modern pig as a result of unintended intense selection pressure for specific carcass and production traits (14).

The relative THW of a pig decreases with increasing BW as the pig ages (1,11,14). Calculating the THW/BW ratio adjusts for these changes and may indicate cardiac hypertrophy. However, the THW/BW ratio cannot differentiate between LV + S, RV, or biventricular hypertrophy (17). Use of the LV + S/RV ratio may provide further insight. Values under 2.8 are reported to indicate RV hypertrophy, and values over 4 indicate LV + S hypertrophy in mature animals (16). Normal-weight reference values for pig hearts could only be found for the measures of THW, THW/BW, and LV + S/RV.

Average values for THW and THW/BW ratio for all categories of hearts in this study were higher than those reported for normal swine hearts in 2 other studies (6,18). However, both of those studies used pigs with an average BW of less than 103 kg, an age of 6 to 12 mo, and a “normal” heart, as defined by the absence of the lesions of hypertrophic cardiomyopathy: dilated LV, LV hypertrophy, cellular disorganization, and medial hyperplasia of the intramural coronary arteries (6,18). The THW/BW ratios listed by Wiseman et al (19) for pigs weighing 125 kg are higher than those found in any heart category in our study. However, the previous article did not describe how the hearts were trimmed before weighing and did not state whether the hearts were examined for lesions. It is possible that a large percentage of the hearts examined in that study had gross lesions of cardiac remodeling that increased the THW/BW ratio. The reference value and range for a normal THW/BW ratio given in a reference text (16) are higher than the mean THW/BW ratio for any heart category in our study, but that text does not provide ages, body weights, or definition of normal, and only 8 pigs were used to establish those values. The mean LV + S/RV values for the hearts in our study indicated RV hypertrophy in the “lesions” and “gross lesions” categories and no hypertrophy in the “lesion-free” category according to the definitions provided in the reference text.
The higher average body weight of pigs with gross and histologic cardiac lesions compared with pigs that do not have gross or histologic lesions implies an increased risk of cardiac remodeling in heavier pigs. Heavier pigs could represent animals with greater than average growth rates affecting both skeletal and cardiac muscle. Studies by Cliplef and McKay (20) as well as Wiseman et al (19) demonstrated that pigs selected for increased growth rate and increased lean gain per day had concurrent increases in heart weight; however, no pathological examination of the heart was done. Alternatively, pigs with greater body weight could be of increased age and have had a longer time to compensate for cardiac insufficiency through the process of cardiac remodeling. Average daily gain and number of days to market were not recorded in our study.

The increased THW/BW and RV/BW ratios for the “lesions” category of hearts compared with those ratios for the other 2 categories are biologically plausible. The relationships between cardiac remodeling and increased heart size (13) and increased cardiac mass with hypertrophy are well-established (17). Heart weights more accurately reflect hypertrophy than wall thickness, particularly eccentric hypertrophy (16). However, THW/BW ratios are not a sensitive test of cardiac remodeling, as small changes in ventricular weight may not be detected (17). This is supported by the overlapping ranges of the THW/BW ratios for the 3 categories of heart in our study.

The lack of significant differences in the THW/BW, LV + S/BW, and RV/BW ratios between the “lesion-free” and “no gross lesions” categories may be because weight changes associated with the histologic lesions of interstitial fibrosis, medial hyperplasia, and perivascular fibrosis of the coronary arteries are likely to be negligible. However, we were unable to find literature describing the effect of histologic lesions on cardiac weight.

In conclusion, the majority (75%) of swine hearts collected from the processing line of an Ontario abattoir had gross and histologic lesions indicating cardiac remodeling. The remodeling process affected cardiac weights and weight ratios, the cardiac weights and the ratios THW/BW and RV/BW being significantly increased in the hearts with gross cardiac lesions. However, further age-specific and weight-specific reference ranges would be required for accurate use of cardiac weights and ratios as predictors of cardiac abnormalities in swine. The large percentage of hearts with cardiac remodeling from clinically healthy market pigs demonstrates an increased probability that subclinical cardiac abnormalities may exist in research pigs, especially if accessed through commercial channels. Researchers should be aware of this likelihood if subclinical cardiac abnormalities could adversely affect their experimental findings. The cause and implications of these lesions in modern pig production should be further investigated.

Acknowledgments

The authors thank Dr. Peter Physick-Sheard, University of Guelph, Guelph, Ontario, for his consultations early in the project regarding data collection. The authors also thank the procurement staff at the abattoir and the postmortem room staff of the Animal Health Laboratory, University of Guelph, Guelph, Ontario, for assistance in the collection, preparation, and storage of the hearts until examination, without which the project would not have been a success. This project was funded through Ontario Pork’s Research Funding (grant 06/13) and through the Agricultural Adaptation Council’s Ontario Farm Innovation Project Funding (grant 0012). Support for Dr. Rothschild was provided by the State of Iowa and Hatch funding.

References


Effects of a single intravenous bolus injection of alfaxalone on canine splenic volume as determined by computed tomography

Michelle M.M. Hasiuk, Fernando L. Garcia-Pereira, Clifford R. Berry, Gary W. Ellison

Abstract

The purpose of this study was to evaluate the effects of a single intravenous dose of alfaxalone on canine splenic volume. In 6 adult beagle dogs the splenic volume [mean ± standard error (SE)] was determined by computed tomography to be 0.17 ± 0.02 L before alfaxalone administration and 0.24 ± 0.02 L (P = 0.0091) and 0.23 ± 0.02 L (P = 0.0268) 15 and 30 min, respectively, after alfaxalone administration. Hematocrits (mean ± SE) obtained at the same times were, respectively, 46.3% ± 1.3%, 40.6% ± 1.3% (P = 0.0015), and 41.7% ± 1.3% (P = 0.0057). In conclusion, alfaxalone caused relaxation of the canine splenic capsule and an increase in the splenic volume, along with a decrease in the hematocrit in these dogs.

Résumé

Le but de cette étude était d’évaluer les effets d’administration intraveineuse d’alfaxalone intraveineuse sur le volume splénique canin déterminé par la tomodensitométrie. Le volume de rate de 6 chiens beagle adultes a été déterminé par tomodensitométrie avant et après l’administration d’alfaxalone. Le volume splénique moyen (± erreur type) était 0,17 ± 0,02 L avant l’administration d’alfaxalone et 0,24 ± 0,02 L (P = 0,0091) et 0,23 ± 0,02 L (P = 0,0268) à 15 min et à 30 min après l’administration d’alfaxalone, respectivement. L’hématocrite moyen (± erreur type) était 46,3 % ± 1,3 % (SEM) avant l’administration d’alfaxalone et 40,6 % ± 1,3 % (P = 0,0015) et 41,7 % ± 1,3 % (P = 0,0057) à 15 min et à 30 min après l’injection. En conclusion, dans cette étude, l’alfaxalone a provoqué une relaxation de la capsule splénique canine et une augmentation de son volume avec une diminution de l’hématocrite.

Introduction

Causes of naturally occurring splenomegaly in dogs include splenic hypertrophy, portal hypertension, infection, primary or infiltrative neoplasia, congestion or enlargement of the spleen due to metabolic disorders, and immune-mediated disease (1–5). Anesthetic drugs can also have significant effects on various vital parameters such as the hematocrit and on organ systems when administered to dogs (3). Some anesthetics and sedatives can cause splenomegaly after their administration (1–3,6). Since enlargement of the spleen is often the primary indicator of splenic disease, drug-induced splenomegaly could result in intraoperative misdiagnosis (1,3). Drug-induced splenomegaly could also complicate surgical approaches to the abdomen, limit exposure of abdominal organs, and increase the risk of accidental splenic laceration, resulting in additional hemorrhage and surgical complications (1,6).

The exact mechanism of changes in splenic size secondary to anesthetic drug administration in dogs is not known but is suggested to be secondary to changes in smooth muscle tone and changes in systemic blood pressure and cardiac output, which alter blood flow (1–3,7). Smooth muscle relaxation, as seen with various anesthetics and sedatives, is suspected to result in relaxation of the muscle fibers in the splenic capsule (1,3). This could permit secondary engorge-
positioning and the location selected for measurement of splenic size (2). However, CT rapidly provides radiographic images and allows for 3-dimensional reconstruction, which is considered the most accurate way to measure organ volume \textit{in vivo} (1,5,12–14). Also, this technique allows for baseline measurement in the awake animal before any drug administration. The area of an organ can be determined on each CT slice and then multiplied by the slice thickness to determine the volume. The sum of the volumes of all the slices reflects organ volume (1,13).

Alfaxalone (3α-hydroxy-5α-pregnane-11,20-dione) is a synthetic neuroactive steroid used for the induction of anesthesia by intravenous (IV) administration (15–22). Its use and properties are similar to those of propofol (18,23). Because its cardiovascular effects are similar to those of propofol and it also causes skeletal muscle relaxation \textit{via} GABA receptors in the central nervous system (15–22), we hypothesized that alfaxalone may have similar effects on the canine splenic capsule, resulting in splenomegaly. To our knowledge, the effect of alfaxalone on canine splenic size was unknown. The purpose of this study was to use CT to measure changes in splenic volume caused by alfaxalone when administered as a single IV bolus to healthy dogs.

**Materials and methods**

Six adult purpose-bred beagles, 1 female and 5 male, aged $7.33 \pm 4.13$ y and weighing $11.4 \pm 1.65$ kg (means $\pm$ standard deviations), were included in this prospective study. They were assessed as healthy from the results of physical examination, a complete blood count, and serum biochemical analyses. They had been acclimatized to handling for several months before the study. The dogs were housed in groups in a climate-controlled room with a light cycle of 12 h of light and 12 h of darkness and acclimatized to a plastic dog crate during the week before treatment and data collection. Food was withheld for 12 h before anesthesia and data collection. \textit{Ad libitum} access to water was permitted. The study was approved Animal Use Form (AUF): 201609448 by the Institutional Animal Care and Use Committee, University of Florida, Gainesville, Florida, USA.

On the day of the study a 22-gauge IV catheter was placed in either cephalic vein without prior sedation. The catheter was connected to a 53-cm-long extension line primed with 3 mL of 0.9\% saline (Baxter International, Deerfield, Illinois, USA). The dogs were placed in a narrow crate on the CT table with the extension line extending outside of the crate to allow for drug administration without interaction with the dog. Saline solution (3 mL) was administered and a baseline CT image obtained. Then 4 mg/kg body weight (BW) of alfaxalone (Alfaxan; Jurox Pty, Rutherford, New South Wales, Australia) was administered through the IV catheter and the extension line flushed.

A multislice CT scanner (Aquilion Prime; Toshiba American Medical Systems, Tustin, California, USA) was used to obtain images at baseline and then 10, 15, and 30 min after alfaxalone injection. Images were acquired by a body standard axial filter with the following parameters: slice thickness 3 mm, collimator pitch PF 0.813/HP 65 (Pitch Factor 0.813/Helical Pitch 65 rows) to improve image quality, 120 kV, modulated mA (50 mA on average), and field large enough to obtain images of the entire abdomen. The images

**Figure 1.** Mean $\pm$ standard error in hematocrit measurements and splenic volume as determined by computed tomography in beagles before, 15 min after, and 30 min after intravenous administration of a single bolus of alfaxalone (4 mg/kg BW). *Value differs significantly ($P \leq 0.05$) from the baseline value.
were imported into commercially available software (Mimics ×64, version 14; Materialise, Plymouth, Michigan, USA). The splenic area was manually delineated on each slice by a board-certified veterinary radiologist (C.R.B.). Splenic volume was calculated by the software program.

Blood samples for measurement of the hematocrit were collected at the time of catheter insertion and by direct venous puncture of the contralateral cephalic vein 15 and 30 min after alfaxalone administration. The samples were placed in heparinized capillary tubes and spun in a microcentrifuge at 3325 × g for 12 min. The hematocrit was measured immediately after centrifugation. The values obtained before and at 15 and 30 min after drug administration were compared.

A multiple-comparisons analysis of variance (ANOVA) with post-hoc Student’s t-test for all pairwise comparisons was used to evaluate the drug’s effects on splenic volume and hematocrit. Results were considered significant if the P-value was 0.05 or less. All data were analyzed with commercial statistical software (JMP PRO 13; SAS Institute, Cary, North Carolina, USA). Normality was assessed with use of the Kolmogorov–Smirnov and Shapiro–Wilk tests.

**Results**

The data were normally distributed. The mean splenic volume [± standard error (SE)] significantly increased after alfaxalone administration (Figure 1), from 0.17 ± 0.02 L at baseline to 0.24 ± 0.02 L after 15 min (P = 0.0091) and 0.23 ± 0.02 L after 30 min (P = 0.0268). There was no significant difference in splenic volume between 15 min and 30 min after alfaxalone administration (P = 0.5420).

Alfaxalone also induced a significant decrease in the mean hematocrit (± SE) (Figure 1), from 46.3% ± 1.3% at baseline to 40.6% ± 1.3% after 15 min (P = 0.0015) and 41.7% ± 1.3% after 30 min (P = 0.0057). There was no significant difference in hematocrit between 15 min and 30 min after alfaxalone administration (P = 0.4353).

**Discussion**

In this study, administration of a single IV bolus of alfaxalone resulted in a significant increase in canine splenic volume as measured by CT. To our knowledge, this is the first report of splenomegaly due to alfaxalone administration. Previous studies have observed increases in splenic volume secondary to administration of barbiturates, acepromazine, and propofol (1,6,8). Although there have been previous conflicting reports of the effects of propofol on splenic volume, a recent study also reported a significant increase in splenic volume after administration of a single bolus of propofol (1).

Although the mechanism for the changes in splenic volume induced by propofol remains unknown, those investigators suggested that it may be secondary to blood redistribution after systemic hypotension paired with the direct effects of the drug on smooth muscle, potentially resulting in relaxation of the splenic capsule and subsequent splenomegaly (1). Alfaxalone administration results in a dose-dependent decrease in arterial blood pressure, which could also lead to blood redistribution, as observed with propofol (19). Barbiturates, such as thiopental, are known to result in splenomegaly, which may be secondary to the same depressive effect on systemic arterial blood pressure due to vasodilation and smooth muscle relaxation (11).

Additionally, propofol has an antagonistic effect on α-adrenergic receptors (24), and perhaps this is the mechanism of splenomegaly seen after administration of propofol, thiopental, alfaxalone, and acepromazine. Acepromazine is a known α-α-adrenergic antagonist that causes effects similar to those of alfaxalone observed in our study. Acepromazine causes a decrease in vascular tone and hematocrit, as well as splenic enlargement (25). Although it is not known if alfaxalone can act directly on the splenic capsule, we hypothesize that its administration caused an increase in splenic volume and a decrease in hematocrit by depressive mechanisms acting on vascular and nonvascular smooth muscle similar to those reported for propofol, thiopental, and acepromazine.

Administration of a single IV bolus of alfaxalone also resulted in a significant decrease in the hematocrit, the first such report, to our knowledge. Previous publications reported a reduction in hematocrit after propofol administration (1,26), and others have attributed the reduction in hematocrit after thiopental administration to be secondary to splenic relaxation and erythrocyte sequestration in the spleen (8,27). Baldo et al (1) observed a significant decrease from baseline in the hematocrit after propofol administration as well as after the administration of acepromazine and thiopental. Although the observed changes were not clinically significant in healthy patients, they could further compromise anemic patients.

Our study was intended to look at the effect of a single IV dose of alfaxalone on the size of the spleen, but other factors can affect this organ. Transport and exposure to the CT room may have resulted in excitement and stress before drug testing. Stress increases the release of adrenaline and noradrenaline, which can result in splenic contraction (28). To reduce this possibility, we ensured that the dogs used in this study had been acclimatized to handling for several months and were acclimatized to the dog crate over the course of a week before the study. Increases in stress hormone levels have been associated with procedures such as venipuncture and catheter placement (28). Release of these hormones can occur rapidly and could have reduced splenic volume before testing (28). However, sedation before catheterization would have added an unwanted variable. The dogs were permitted to recover after injection of the single bolus of alfaxalone. Excitement during emergence from anesthesia, identified as paddling and dysphoric vocalization, was observed around 20 min after alfaxalone administration in all the dogs. This excitement could have resulted in increased release of adrenaline and noradrenaline, which may have limited the full effect of alfaxalone on splenic size and caused the decrease in size at 30 min. Therefore, it is not certain if alfaxalone reached its maximum effect on splenic size and whether the results would have been different if anesthesia had been maintained with a continuous infusion of the drug until the CT scan at 30 min. Restraint would have introduced another variable and might have exacerbated the stress during recovery. No injuries occurred during recovery and no treatment was needed.

The dogs tolerated being confined to the crate during CT image acquisition; however, the crate did allow the dogs mobility within the crate. If considerable motion was observed on review of the CT images immediately after acquisition, CT was done again until images acceptable for accurate determination of splenic volume
were obtained. All studies were completed within 4 min of the designated time. The longer time required to acquire the images and the motion may have limited the full extent of splenomegaly because of drug metabolism and increased catecholamine levels during the recovery phase.

Use of CT for determination of organ volume has been found to be reliable and accurate (5,13). When ultrasonography is used, patient position and operator can affect the measurement of visceral volume. A study comparing ultrasonography with CT found the latter to be superior in measuring organ volume. However, even using CT, hand-outlining of organs requires dexterity and expertise to accurately delineate the organ when there are edges of low contrast (5). For this reason, outlines of the spleens in our study were done by a single Board-certified radiologist. A previous study, however, found low interobserver and intraobserver variability in the evaluation of organ volume from CT images (13).

In this study, administration of a single IV bolus of alfaxalone to healthy dogs resulted in significant splenic enlargement and reduction in hematocrit. These changes may not be clinically significant in healthy dogs but may compromise animals with such conditions as anemia and diaphragmatic herniation. Also, the increase in splenic size can make it easier to puncture the spleen with a trocar during laparoscopic procedures. It is unknown whether these effects would be maintained while the patient is under general anesthesia and whether these changes would persist until the time of surgical exploration. It is also unknown whether these effects would be observed with different alfaxalone dosages or in drug combinations. Furthermore, the impact on and clinical implications for subsequent anesthesia maintenance with inhalant anesthetics after induction with alfaxalone are unknown. In addition, conditions that may affect splenic size, including neoplasia, rickettsial diseases, and high sympathetic output, may alter the effects of alfaxalone.

In conclusion, a single IV bolus of alfaxalone produced splenic enlargement and decreased hematocrit in our canine population. Therefore, alfaxalone should not be administered if splenomegaly is to be avoided.

Acknowledgments

The authors thank Ms. Mary Wilson, Ms. Christine Fitzgerald, Ms. Rachel Sanford, Ms. Kim Ahrens, Dr. Rosanna Marsella, and Mr. Andrew Morris for their invaluable assistance with this project. Funding for the study was provided by Dr. Gary Ellison’s clinical research funds.

References

Intraocular pressure measurements in cattle, sheep, and goats with 2 different types of tonometers

Nina Peche, Johanna Corinna Eule

Abstract

The aim of this study was to investigate normal intraocular pressure (IOP) values of cattle, sheep, and goats with a rebound tonometer (TonoVet (TV)) and an applanation tonometer (Tono-Pen AVIA (TPA)) and to determine correction functions for the 2 devices. A total of 60 healthy cattle, sheep, and goats (20 of each) underwent slit-lamp biomicroscopy. Intraocular pressure (IOP) readings were taken from both eyes with the 2 different tonometers and statistically analyzed. For calibration purposes, the IOP was preset on each instrument at 5 to 60 mmHg using 5 mmHg increments in 10 bovine, 8 ovine, and 6 caprine freshly enucleated eyes. Readings were taken with both tonometers at each interval and compared to the manometrically controlled IOP (Mann-Whitney U-test, \( P \leq 0.05 \); Bland-Altman plot, and regression analysis). The median IOP measurements (min to max) obtained with the TV were 23 mmHg (12 to 40 mmHg), 11 mmHg (7 to 20 mmHg), and 23 mmHg (9 to 37 mmHg) for cattle, sheep, and goats, respectively. Using the TPA, the median IOP measurements were 16 mmHg (8 to 27 mmHg), 10 mmHg (5 to 18 mmHg), and 13 mmHg (4 to 25 mmHg) for cattle, sheep, and goats, respectively. There were statistically significant differences between the readings taken with the TV and the TPA in all species (Wilcoxon-test, \( P \leq 0.05 \)). All measurements obtained with the TV and the TPA during the calibration procedure differed statistically significantly from the manometrically controlled IOP measurements (Mann-Whitney U-test, \( P \leq 0.05 \)). For both instruments, regression formulas were calculated to correct the measurements. Both tonometers can be used effectively to assess intraocular pressure in ruminants, using the specific regression formulas.

Résumé

L’objectif de cette étude était de mesurer la pression intraoculaire (PIO) normale chez la vache, le mouton et la chèvre à l’aide de deux tonomètres différents; le (TonoVet (TV)) et le Tono-Pen AVIA (TPA) et de déterminer les fonctions de correction pour ces deux appareils. Les vingt animaux de chaque espèce ont été examinés à la lampe à fente pour être qu’ils ne présentaient aucune anomalie oculaire puis la PIO des deux yeux fut mesurée avec les deux tonomètres différents et les valeurs furent analysées statistiquement. Pour des raisons d’étalonnage la PIO fut préréglée de 5 à 60 mmHg, divisée en incrément de 5 mmHg sur 10 yeux de vaches, 8 yeux de moutons et 6 yeux de chèvres fraîchement énucléées. Pour chaque incrément de pression, les lectures furent prises avec les deux tonomètres et furent comparées à la PIO mesurée manométriquement. Chez les bovins, les ovins et les caprins la PIO médiane (min-max) obtenue avec le TV fut de 23 mmHg (12-40 mmHg), 11 mmHg (7-20 mmHg) et 23 mmHg (9-37 mmHg) et avec le TPA de 16 mmHg (8-27 mmHg), 10 mmHg (5-18 mmHg) et 13 mmHg (4-25 mmHg). Dans toutes les espèces, des différences statistiquement significatives entre les lectures prises avec la TV et la TPA furent constatées. Lors de la procédure d’étalonnage toutes les valeurs de la PIO mesurées avec le TV et le TPA furent statistiquement significativement différentes des valeurs obtenues manométriquement. Afin de corriger les mesures les formules de régression furent calculées pour les deux instruments. En tenant compte des formules de régression spécifiques les deux tonomètres peuvent être utilisés efficacement pour évaluer la pression intraoculaire chez les ruminants.

Introduction

Normal and relatively constant intraocular pressure (IOP) is maintained by the balance of aqueous humor production and outflow. This relatively constant pressure is essential in order to maintain the shape of the eye and to ensure a stable position of the intraocular structures (1,2). Intraocular pressure (IOP) should be measured in each patient presented for ophthalmic examination, as tonometry is essential for diagnosing and monitoring uveitis and glaucoma. The most accurate method of measuring IOP is direct tonometry using a manometer, but this procedure is not practical for clinical use due to its invasiveness. In current clinical veterinary ophthalmology, application and rebound tonometry are the most widely used techniques for measuring IOP. Both methods indirectly determine IOP by measuring corneal tension (3).

There are many published studies that compare the different methods and available instruments for measuring IOP in various animal species. In veterinary ophthalmology, the Mackay-Marg application tonometer has been widely used in the past and has been shown to have good accuracy. It seems to be the most reliable device for measuring IOP in different animal species, both with and without ocular abnormalities. The accuracy and usefulness

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of the commercially available Tono-Pen XL, the Perkins handheld tonometer, and the TonoVet (TV) have been extensively studied in different animal species (4–15).

To accurately measure pressure, each type of tonometer must be calibrated for use with different animal species as ocular anatomy, e.g., corneal thickness and curvature, corneal and scleral rigidity, and viscosity of tear film, varies among the species. Calibration curves are therefore necessary for the different tonometers. Although several studies are available in dogs, cats, horses, cows, sheep, rats, mice, and chinchillas (3,11,15–18), there are no calibration studies for the TV and newly designed Tono-Pen AVIA (TPA) in domestic ruminants.

Depending on the instrument used, the mean IOP reported for cattle, sheep, and goats ranged from 15 to 28 mmHg, 11 to 14 mmHg, and 11 to 12 mmHg, respectively (Table I) (5,6,12,19–22). Although the incidence of glaucoma in cattle is very low, even less than 1%, various ocular diseases (inflammatory or neoplastic conditions) can lead to alterations in production of aqueous humor and outflow, which results in an increased or decreased IOP (23–25). Ocular hypertension has been experimentally induced in cattle and sheep using topical application of corticosteroids (21,26).

To the authors’ knowledge, no studies are currently available that compare applanation and rebound tonometry or calibration curves for use of the TV or the TPA in ruminants. The aim of this study was to investigate normal IOP values in cattle, sheep, and goats using a rebound tonometer (TonoVet) and an application tonometer (Tono-Pen AVIA) and to determine regression formulas for the 2 devices.

Materials and methods

Patient examination

A total of 60 healthy, privately owned cattle, sheep, and goats (20 of each) was examined individually at the owner’s request as part of a general health check. All procedures were conducted according to the guidelines of the Association for Research in Vision and Ophthalmology (ARVO). The animals were examined in their normal environment to keep the stress level as low as possible. The cows were restrained in their feed fence with the head fixed with a halter to either the right or left side to avoid any pressure on the jugular vein. All sheep were positioned on their backside as routinely done for shearing and their heads were gently held straight ahead. Goats were kept in a standing position with their heads restrained by their horns, looking straight ahead.

All 60 animals underwent slit-lamp biomicroscopy of the anterior segments of both eyes using a Kowa-SL 15 (Kowa, Tokyo, Japan). The intraocular pressure of each eye was initially measured with the TonoVet (Tiolat Oy, Helsinki, Finland) using the “d” setting. After rebound tonometry, 2 drops of the local anesthetic agent oxybuprocaine hydrochloride (Novesine 0.4%; OmniVision, Puchheim, Germany) were applied to both eyes. Thirty seconds after instillation of this medication, the Tono-Pen AVIA (Reichert Technologies, Depew, New York, USA) was used to take IOP readings from each eye. Triplicate readings were taken for each eye with both devices and then readings were averaged. The eye to evaluate first was randomly selected. Care was taken to open the eyes without applying any pressure on the globe.

Manometric examination

Before the manometric examination, the manometer (D D-890; ATP Messtechnik, Ettenheim, Germany) was checked by the Bureau of Standards of the Federal States of Berlin and Brandenburg, Germany.

Ten bovine, 8 ovine, and 6 caprine freshly enucleated eyes from slaughtered animals were used for the ex-vivo measurements. After enucleation, the eyes were immersed in a 0.9% sodium chloride (NaCl) solution and stored at room temperature. All measurements were conducted within 6 h after enucleation.

For the manometric experiments, the enucleated eyes were placed on a bed of modeling material on top of a plastic cup to avoid any pressure or movement during the examination. The eyes were cannulated through the sclera into the vitreous cavity with a 23-gauge needle. The needle was connected to the manometer and to a saline

Table I. Published values for intraocular pressure (IOP) in ruminants using different tonometers.

<table>
<thead>
<tr>
<th>Study</th>
<th>Species and breed</th>
<th>Number of animals (N)</th>
<th>Mean IOP ± SD (mmHg)</th>
<th>Tonometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gum et al, 1998 (12)</td>
<td>Cattle (Holstein-Fresian, Jersey)</td>
<td>32</td>
<td>27.5 ± 4.8</td>
<td>Mackay-Marg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>28.2 ± 4.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>26.9 ± 6.7</td>
<td>Tono-Pen XL</td>
</tr>
<tr>
<td>Kotani, 1993 (19)</td>
<td>Cattle</td>
<td></td>
<td>23.4 ± 5.9</td>
<td>Mackay-Marg</td>
</tr>
<tr>
<td>Andrade et al, 2011 (5)</td>
<td>Horses and cattle</td>
<td>10</td>
<td>18.8 ± 1.7</td>
<td>Perkins</td>
</tr>
<tr>
<td>Tofflemire et al, 2015 (20)</td>
<td>Cattle calves (Holstein)</td>
<td>33</td>
<td>15.2 ± 5.2</td>
<td>TonoVet</td>
</tr>
<tr>
<td>Gerometta et al, 2009 (21)</td>
<td>Sheep (Corriedale)</td>
<td>18</td>
<td>10.6 ± 1.4</td>
<td>Perkins</td>
</tr>
<tr>
<td>Ribeiro et al, 2014 (22)</td>
<td>Sheep (Santa Ines)</td>
<td>10</td>
<td>OS 12.70 ± 1.09</td>
<td>Tono-Pen XL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OD 13.90 ± 0.84</td>
<td></td>
</tr>
<tr>
<td>Broadwater et al, 2007 (6)</td>
<td>Goats (Pygmy)</td>
<td>10</td>
<td>11.8 ± 1.5</td>
<td>TonoVet</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.8 ± 1.7</td>
<td>Tono-Pen XL</td>
</tr>
</tbody>
</table>

SD — standard deviation; OS — oculus sinister; OD — oculus dexter.
solution reservoir via a 3-way stopcock. An open system was used for all measurements. The IOP was sequentially increased from 5 to 60 mmHg in increments of 5 mmHg by adjusting the height of the saline reservoir. Minimal changes in the manometrically controlled IOP (± 0.1 mmHg) were tolerated, but higher differences were corrected immediately. There was no leakage of fluid observed around the needles during any measurements.

The rebound tonometer was always used first and the applanation tonometer second. To keep the cornea moist throughout the whole examination, 4 to 5 drops of saline were applied to its surface before each measurement. For each interval, 6 consecutive measurements were taken with both devices. In each measurement series, the lowest and the highest value were excluded from statistical analysis. All values were compared to the manometrically determined pressure.

The Statistical analysis

The statistical software R (version 3.1.0) was used for the statistical analysis. Data were tested for a normal distribution with the Shapiro-Wilk test. The Wilcoxon test was conducted to detect differences between the measurements of the TV and the TPA for the right and left eye measurements. The obtained values were not normally distributed. The results at each IOP level were statistically significant from the manometrically determined pressure by 5 to 25 mmHg and overestimated it by 30 to 60 mmHg. The TPA underestimated the pressure throughout the whole range of IOP levels (Figures 1a, 2a, 3a, and 4a). In sheep and goats, both instruments underestimated the manometric pressure by 5 to 60 mmHg (Figures 1b, c; 2b, c; 3b, c; and 4b, c). To correct the measured results, regression formulas were calculated for the 2 tonometers for all 3 species (Table III). Differences between the left and right eyes were statistically significant for the measurements using the TV in cattle and goats.

Table II. Results of intraocular pressure measurements [median, min to max, mean ± standard deviation (SD)] and corresponding P-values in cattle, sheep, and goats using the TonoVet and Tono-Pen AVIA.

<table>
<thead>
<tr>
<th>Eye</th>
<th>TonoVet Median (min to max)</th>
<th>Tono-Pen AVIA Median (min to max)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle OD 23 (15 to 37)</td>
<td>15.5 (8 to 27)</td>
<td>23.9 ± 5.0</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>22 (12 to 40)</td>
<td>16 (9 to 25)</td>
<td>21.5 ± 6.3</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>Sheep OS 11 (8 to 20)</td>
<td>10 (5 to 18)</td>
<td>12.7 ± 3.0</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>10.5 (7 to 20)</td>
<td>10 (6 to 18)</td>
<td>11.7 ± 3.3</td>
<td>0.009</td>
</tr>
<tr>
<td>Goats OS 22 (9 to 34)</td>
<td>13 (4 to 25)</td>
<td>21.6 ± 5.4</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>24 (11 to 37)</td>
<td>13 (6 to 25)</td>
<td>24.3 ± 5.6</td>
<td>&lt; 0.000</td>
</tr>
</tbody>
</table>

Table II. Differences between the left and right eyes were statistically significant for the measurements using the TV in cattle and goats.

### Results

**Clinical tonometry**

A total of 120 eyes from 60 healthy domestic ruminants (20 cows, 20 sheep, and 20 goats) was examined. All cattle were Holstein-Friesian dairy cows with a median age of 4 y (2 to 10 y). The sheep were German heaths from a hobby breeder. Eleven animals were female and 9 were male, both with a median age of 0.5 y old (0.5 to 12 y). The goats were all female Toggenburgers and were kept for milk production and reproduction. They were a median age of 6.5 y (2 to 14 y). Based on the ophthalmic examination, all animals were free of ocular disease.

Fresian dairy cows with a median age of 4 y (2 to 10 y). The sheep were German heaths from a hobby breeder. Eleven animals were female and 9 were male, both with a median age of 0.5 y old (0.5 to 12 y). The goats were all female Toggenburgers and were kept for milk production and reproduction. They were a median age of 6.5 y (2 to 14 y). Based on the ophthalmic examination, all animals were free of ocular disease.

The obtained values were not normally distributed. The results of the intraocular pressure measurements [median (min to max), mean ± standard deviation (SD), and P-values] are listed in Table II. There were statistically significant differences between the measurements obtained with the TV and the TPA in all 3 species, as shown in Table II. Differences between the left and right eyes were statistically significant for the measurements using the TV in cattle and goats.

### Discussion

An adequate interpretation of IOP values requires reference values for the particular measuring device and the relevant species. Tonometers therefore need to be calibrated for use in different species, as ocular anatomy varies among different animal species.
Figure 1. Bland-Altman plots for cattle (a), sheep (b), and goats (c) showing that the TonoVet tends to underestimate and then overestimate the true intraocular pressure (mmHg).

Figure 2. Bland-Altman plots for cattle (a), sheep (b), and goats (c) showing that the Tono-Pen AVIA underestimates the true intraocular pressure (mmHg) over the whole pressure range.
Figure 3. Regression analysis for the TonoVet in cattle (a), sheep (b), and goats (c). Calculated regression line (solid line) and ideal regression line (interrupted line).

Figure 4. Regression analysis for the Tono-Pen AVIA in cattle (a), sheep (b), and goats (c). Calculated regression line (solid line) and ideal regression line (interrupted line).
Table III. Calculated regression formulas and corresponding \( r^2 \) values for 2 tonometers used in cattle, sheep, and goats to correct measured values.

<table>
<thead>
<tr>
<th></th>
<th>TonoVet</th>
<th>Tono-Pen AVIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>( y = 1.226x - 5.392 )</td>
<td>( y = 0.7141x - 0.7864 )</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.98</td>
<td>0.92</td>
</tr>
<tr>
<td>Sheep</td>
<td>( y = 0.9816x - 2.5601 )</td>
<td>( y = 0.6337x - 1.1840 )</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.98</td>
<td>0.98</td>
</tr>
<tr>
<td>Goats</td>
<td>( y = 1.047x - 5.0551 )</td>
<td>( y = 0.6476x - 3.0905 )</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.97</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Our IOP readings in cattle using the TV were almost consistent with the values obtained in a previous study using the Mackay-Marg tonometer (19) and were slightly lower than those reported in a study using the Mackay-Marg tonometer and the Tono-Pen XL (12). The values measured with the TPA in our study were quite similar to those reported in a study using the Perkins handheld tonometer (5). Another study using the Perkins tonometer found a mean IOP (± SD) of 10.6 ± 1.4 mmHg in sheep, which is quite similar to our measurements taken with the TPA (21). Our measurements with the TV revealed slightly higher results, which were comparable to those from a recent study using the Tono-Pen XL (22).

Only 1 study using the Tono-Pen XL and the TV established reference values for IOP measurements in goats (6). The results in that study were quite similar to the values we obtained with the TPA. We found significantly higher values for the IOP in goats measured with the TV. This may be due to differences in breeds, different fixation methods, or diurnal variation. All the cattle and goats in our study were females, but gender is not reported to have a significant influence on IOP in several other species (6,27,28).

In contrast to previous studies in which no differences were detected between the left and right eyes (6,12,14,20,22), we found statistically significant differences between the left and right eyes in cattle and goats, but only for the measurements made with the TV. As the eye measured first was always randomly selected, the order of measuring cannot account for this phenomenon. As this difference between eyes was only detectable in cattle and goats and only with the use of the TV, an examiner-related cause seems unlikely. The reason for these statistically significant differences remains unknown. The difference between eyes is only up to 2 mmHg, however, and may not be of any clinical importance. This difference may no longer be significant in a study with a larger number of animals.

The TV offers 3 settings (“h” for horse, “d” for dog, and “p” for other species) for evaluating the intraocular pressure in different species to account for various globe sizes and anatomic variations. In the clinical part of our study, the “h” setting was initially used in cows (\( n = 10 \)), as it was assumed that the bovine globe was most similar to the equine globe. As readings without error were obtained in only 2/10 animals, the “d” setting was tried and evaluable readings were obtained in 10/10 animals. Similar issues were discussed in another study, in which it was stated that the most accurate setting for use in cattle is unclear (20). The manometric results of our study showed that the “d” setting can be used for tonometry in cattle, sheep, and goats.

Although glaucoma is rare in ruminants, tonometry is still an important part of the ophthalmic examination. Both instruments (the TV and the TPA) used in this study are handheld devices that can be easily transported and used in mobile food animal practice. Measurements made with the TV appeared to be harder to obtain in cows due to the difficulty of holding their heads in an appropriate position. The TV needs to be held in a perpendicular position to the cornea with the tip parallel to the ground, whereas the TPA can be used independently of the head position.

Comparing the 2 tonometers, we found statistically significant differences in all 3 species. We always measured with the rebound tonometer (TonoVet) first because the measurements can be taken without the use of a topical anesthetic agent. Some authors have reported that a tonographic effect of a rebound tonometer is unlikely, assuming that the order of tonometer application does not affect the IOP results when using a rebound tonometer before an applanation tonometer (11,29). Another study showed that the Tono-Pen significantly underestimated the pressure in normal, healthy cat eyes in vivo compared to the Mackay-Marg tonometer (10). Interestingly, with regard to the order in which the instruments are used, different values were obtained with the Tono-Pen when used after the Mackay-Marg tonometer. Furthermore, this study found that both instruments tended to underestimate the pressure in open and closed in-vitro systems in cat eyes compared to direct manometry (10). No significant differences were found between the 2 different applanation tonometers in studies of in-vivo measurements using either the Mackay-Marg or the Tono-Pen XL in horses and cows, respectively (9,12).

Compared with manometry in freshly enucleated eyes, all values from both tonometers, except for 1 using the TPA in sheep eyes, differed significantly from the manometrically set IOP. We excluded any tonographic effect, at least for the manometric study, by immediately adjusting the saline reservoir if pressure changes exceeded 0.1 mmHg.

In general, there were different results regarding the over- or underestimation of rebound tonometers. The underestimation of IOP by 5 to 25 mmHg and overestimation by 30 to 60 mmHg in cattle eyes using the TV in our study is consistent with a previous study in cats (30). In sheep and goat eyes, we found an overall underestimation with the TV. Another study found a good agreement for the TV in enucleated dog eyes throughout the whole pressure range (5 to 80 mmHg), whereas in enucleated horse eyes, the TV significantly underestimated the IOP for pressures greater than 70 mmHg (31). Another study in horses found that the TV tended to slightly overestimate IOP in the clinically relevant pressure range from 10 to 60 mmHg, while underestimating the true IOP for pressure values greater than 70 mmHg (18).

The overall underestimation that occurred when using the TPA in our study is consistent with the findings for applanation tonometers in dogs and cats (11,15). In a calibration study, the TonoPen XL underestimated the true IOP in cows and sheep mainly at high settings (15). In another study, 2 applanation tonometers (the Mackay-Marg and the Tono-Pen) also underestimated IOP significantly compared to direct manometry in enucleated cat eyes in open and closed in-vitro systems (10). In equine eyes, neither the Mackay-Marg nor the Tono-Pen calculated IOP accurately compared to the manometric
measurements (9). The Tono-Pen consistently overestimated IOP at lower pressure levels and underestimated IOP at higher pressure levels compared to manometric measurements in the previously mentioned study in cow and sheep eyes (15), whereas other studies in different species found that overall, the true IOP was underestimated (10,11,15).

In all 3 species, the rebound tonometer (TonoVet) provided more accurate results for IOP than the application tonometer (Tono-Pen AVIA), which is in agreement with another recent study in cat eyes (8). In contrast to our study, however, the IOP values measured in this study using the TV were consistently slightly higher than the manometrically controlled IOP in the cat eyes (8).

Although we found high $r^2$ values in most cases, it must be recognized that there is a significant difference between the manometrically set IOP and the measured IOP with both tonometers, which is in accordance with a previous study (11). We calculated regression formulas to correct the measured values in order to obtain reliable values. For daily clinical use, a simple correction factor would have been more suitable.

The limitations of our study were the small number of subjects and the fact that only one breed of each species was examined. Furthermore, we did not measure the central corneal thickness, which could be a possible source of error (32,33).

In conclusion, our study established additional reference values for measuring IOP in ruminants. Our results show the importance of calibrating every tonometer for each species. The same type of tonometer should always be used for surveillance of clinical patients. It should also be remembered that application tonometers, such as the Tono-Pen AVIA, tend to underestimate the true IOP, especially at higher pressure levels. While the TonoVet (TV) offered much more reliable results, however, it was more difficult to use with cows. In general, both tonometers, TonoVet (TV) and Tono-Pen AVIA (TPA), can be used effectively to assess intraocular pressure in ruminants, using the specific regression formulas.

Acknowledgment

The authors thank Florian Peche (BSc) for support with the statistical analysis of this study.

References


The biological characteristics of sheep umbilical cord mesenchymal stem cells

Fenghao Chen, Chenqiong Zhao, Yuhua Zhao, Li Li, Shi Liu, Zhiqiang Zhu, Weijun Guan

Abstract

Although mesenchymal stem cells (MSCs) are now regarded as a promising cell resource for tissue repair and regeneration, the optimal source of MSCs has not yet been determined. The objective of this study was to provide a theoretical basis for the clinical application of umbilical cord mesenchymal stem cells (UCMSCs) in the future. Umbilical cord is an easily obtainable tissue resource, which is one reason that it has become a candidate resource for mesenchymal stem cells. In this study, we analyzed the biological characteristics of UCMSCs, such as their multiple differentiation and clone-forming ability, through morphological observation, reverse transcription polymerase chain reaction (RT-PCR), growth curve, positive rate test, and immunophenotype. Umbilical cord MSCs were successfully isolated and passaged to 29 generations. The results from RT-PCR showed that UCMSCs were positive for CD29, CD44, CD73, but negative for CD34. The expression of the stem cell marker nucleostemin and tenocyte-related markers showed similar positive results with CD44, CD73, and CD90. In addition, UCMSCs can be induced to differentiate into osteoblasts, adipocytes, or chondrocytes. Our study showed that UCMSCs not only have the ability to self-renew, but also have the potential to differentiate into multiple lineages. In general, we concluded that UCMSCs are a reliable source for use in cell therapy.

Résumé

Bien que les cellules souches mésenchymateuses (CSMs) soient maintenant considérées comme une ressource promise pour cellules pour la réparation tissulaire et la régénération, la source optimale des CSMs n’a pas encore été déterminée. L’objectif de la présente étude était de fournir une base théorique pour l’application clinique de cellules souches mésenchymateuses de cordon ombilical (CSMCO) dans le futur. Le cordon ombilical est une ressource tissulaire pouvant être obtenue facilement, une des raisons pour laquelle il est devenu un candidat pour les CSMs. Dans cette étude nous avons analysé les caractéristiques biologiques des CSMCO, telles que leur différenciation multiplex et la capacité à former des clones, par des observations morphologiques, par réaction d’amplification en chaîne par la polymérase avec la transcriptase réverse (ACP-TR), courbe de croissance, test de ratio positif, et immunophénotype. Les CSMCO ont été isolées avec succès et des passages obtenus jusqu’à la 29e génération. Les résultats d’ACP-TR ont montré que les CSMCO étaient positives pour CD29, CD44, CD73, mais négative pour CD34. L’expression de nucléostémine, un marqueur de cellule souche, et de marqueurs apparentés aux ténocytes ont montré des résultats positifs similaires à ceux de CD44, CD73, et CD90. De plus, les CSMCO peuvent être induites à se différencier en ostéoblastes, adipocytes, ou chondrocytes. Notre étude a démontré que les CSMCO ont non seulement la capacité de s’auto-renouveler, mais ont également le potentiel de se différencier en des lignées multiples. En général, nous avons conclu que les CSMCO sont une source fiable pour utilisation en thérapie cellulaire.

Introduction

Mesenchymal stem cells (MSCs) are important components of the microenvironment of sheep. As they are easily amplified, isolated, and cultured in vitro, umbilical cord-derived MSCs have become a popular topic for research in recent years. Umbilical cord is a promising resource for repairing damaged tissues (1). Umbilical cord MSCs are easily obtained animal material and their proliferation and long-term storage are fairly stable, with a low incidence of transplant rejection, graft-versus-host disease, and infection (2). In addition, there are no passive effects. Therefore, umbilical cord mesenchymal stem cells (UCMSCs) not only have pluripotency and polyfunction, but can also be applied to organ regeneration.

Although small-tail Han sheep are an important model in scientific research, the study of mesenchymal stem cells (MSCs) from sheep is still in its early stages. Sheep MSCs are derived from spinal marrow and less immature than the umbilical cord mesenchymal stem cells (3). Unlike embryonic stem cells (ESCs), however, when umbilical cord-derived MSCs were subcutaneously injected into nude mice, no teratomas were generated (4). Umbilical cord MSCs have all the characteristics previously described because they have more immature parental generations than bone marrow mesenchymal stem cells (BMSCs), and umbilical cord MSCs possess lower graft-versus-host disease and infection abilities, and are stable in proliferation and long-term storage (5,6). In addition, the immaturity and low immuinity of UCMSCs are related to the optimum therapeutic efficiency.
to tissues and organs. At present, research into isolated UCMSCs is focused primarily on cellular morphology, certain markers, and the generation and differentiation of cells.

**Materials and methods**

**Experimental animals**

Animal experiments were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences. The Chinese Han sheep used in this study were obtained from the Chinese Academy of Agricultural Sciences. A total of 8 fetuses (3- to 4-months old) were removed by caesarean section for use in this research. Fetus samples were collected, stored in an ice tray, and transported to the laboratory within 4 h after the caesarean section.

**Isolation and culture of UCMSCs**

Fresh umbilical cords were obtained from fetal lambs and the skin and blood vessels were removed. The tissues were then washed with phosphate-buffered saline (PBS) 6 to 8 times to remove red blood cells. Wharton’s jelly tissue was cut into pieces of 1 mm$^3$ with ophthalmic scissors and digested with 3 mg/mL of 0.2% type-II collagenase (Sigma-Aldrich, St. Louis, Missouri, USA) and 0.125% trypsin for 1 h at 37°C in a CO$_2$ incubator (Hersaeus BB5060UV; Shanghai Lishen Scientific Equipment Co., Shanhai, China) (7). The fragments of umbilical cord were passed through a 200-mesh sieve and treated with Dulbecco’s Modified Eagle Medium (DMEM/12; GIBCO, Carlsbad, California, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, California, USA), to terminate the reaction.

The single cell suspension was placed in a centrifuge tube (15 mL) and incubated with 3 mL of DMEM/12 medium containing 10% fetal bovine serum in a family. After being centrifuged (1200 rpm) for 10 minutes, the tube was divided into two layers and the supernatant was discarded. The cells were resuspended in DMEM/12 complete culture medium supplemented with 10% FBS (Invitrogen), 100 U/mL penicillin (Invitrogen), 100 mg/mL streptomycin (Invitrogen), 2 ng/mL EGF (Peprotech, Rocky Hill, Texas, USA), and 2 mM L-glutamine (Invitrogen). The cell suspension was seeded in a 60-mm petri dish (Wuxi Nest Biotechnology, Jiangsu, China) at 1 $\times$ 100 cells/mL and incubated at 37°C with 5% CO$_2$ (7). Culture medium was replaced every 36 h. Cells were digested with 0.25% trypsin and 0.02% EDTA when the convergence rate reached 70% to 80% and passaged into new culture dishes with the ratio of 1:1. Normally, after 3 or 4 passages (P), freshly isolated cells were homogenous and purified.

**Growth kinetics**

To assess growth dynamics of UCMSCs, cells of passage 4 (P4), P14, and P24 were seeded in 24-well plates at a density of

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**Table I. Primer sequences used in the reverse transcription polymerase chain reactions.**

<table>
<thead>
<tr>
<th>Gene names</th>
<th>Primer sequences</th>
<th>TM (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
</table>
| CD29       | F: 5'-TCTCCAGAAAGGCAAAC-3'  
            | R: 5'-GTAACGAACCGAAGCAAAG-3' | 57.8    | 286                 |
| CD73       | F: 5'-CAGGGTTTCTCCAGGAATCG-3'  
            | R: 5'-TCTCTTCAACACGACGATCC-3' | 58      | 304                 |
| CD44       | F: 5'-GCCAGGTTTCTCCATAAGC-3'  
            | R: 5'-TAAACAGAATGTCACGCCAA-3' | 57      | 310                 |
| LPL        | F: 5'-GCGTTCCGCTCATCTCTTT-3'  
            | R: 5'-CTGTTGTTGTTGATGATATTACTCT-3' | 59      | 274                 |
| PPARG      | F: 5'-ATCAAGGTCAGCAGACATCAG-3'  
            | R: 5'-CATCTCAAGGTTACGAC-3' | 60      | 154                 |
| OPN        | F: 5'-AGGTTGTAGTGTGGCTATG-3'  
            | R: 5'-GATGGGAATGCTTGTGCTCT-3' | 58      | 233                 |
| Col-1      | F: 5'-CAGAATGGAAGCAGGTT-3'  
            | R: 5'-GCAATGGTAGGTTGATGTTGTC-3' | 60      | 305                 |
| Sox9       | F: 5'-GTGGCTCAAGGCTACAGTTGG-3'  
            | R: 5'-CGTCTCTACGCTGACTCTCTC-3 | 62      | 362                 |
| Col-2      | F: 5'-CAGGCTCAAGGCTCAGAAG-3'  
            | R: 5'-AAGGAGAAGGTGCTTCTAGTCA-3 | 62      | 323                 |
| GAPDH      | F: 5'-CACTGTCCAGGCACATCCT-3'  
            | R: 5'-CTGTGTTGTTTAGCAGAATT-3' | 55      | 442                 |
3.10^4 cells/well per passage and continually cultured for 8 d. Cells were counted by blood cell-counting instrument (3 wells per time and the mean value of the cell counting was calculated) (Beijing Liuyi Instrument Factory, Beijing, China) (6). After a latency phase of 1 to 3 d, cell growth entered the logarithmic phase and plateaued at around day 7. The population-doubling times (PDTs), using the formula PDT = (t2 - t0) log2/(logNt - logN0), were determined to be 8, 10, and 14 h for P4, P14, and P24, respectively.

**Immunofluorescence analysis**

Umbilical cord MSCs of P4 were seeded in 6 pore plates and then cultured in complete medium. Cells were washed 3 times (5 min each) in PBS until the convergence rate reached approximately 70%. Cells were then fixed with 4% paraformaldehyde at room temperature for 30 min and washed 3 times with PBS. Cells were permeabilized with 0.2% Triton X-100 for 20 min and washed 3 times (5 min each) in PBS. The cells were blocked with 10% normal mouse serum (Santa Cruz Biotechnology, Santa Cruz, California, USA) for 1 h at room temperature. Cells were then extracted out without being washed with PBS. The primary antibodies, rabbit anti-CD44 (1:100; Bioss, Beijing, China), rabbit anti-CD73 (1:100; Bioss), rabbit anti-CD90 (1:100; Bioss), and rabbit anti-CD105 (1:100; Bioss) were added to cells. The cells were then incubated at 4°C overnight. The primary antibody was removed and cells were washed 3 times with PBS. Secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated mouse anti-rabbit (Bioss). The secondary antibody solution was decanted and washed 3 times with PBS in darkness. The cells were then incubated in 1 g/mL DAPI (Sigma-Aldrich) for 15 min and washed 3 times with PBS. Cells were examined under a TE-2000-E Inverted Fluorescence Microscope (Nikon, Yokohama, Kanagawa, Japan).

**Reverse transcriptase-polymerase chain reaction (RT-PCR) assay**

Isolated UCMSs of P4, P14, and P24 were collected and extracted by using TRIzol reagent (Invitrogen). The ribonucleic acid (RNA) concentrations of different passages were measured by absorbance at 260 nm with a spectrophotometer. Complementary deoxyribonucleic acid (cDNA) was synthesized using a reverse transcription PCR system (Takara, Liaoning, China) and amplified by PCR using specific primers (Table I). Polymerase chain reaction (PCR) products were visualized by 20 g/L agarose gel electrophoresis.

**Colony-forming cells assay**

The P4, P14, and P24 cells were seeded in 6-well plates at 1 × 10^4 cells/well and cultured for 7 d. The colony-forming rate was formulated as colony-forming unit numbers/starting cell
number per 6 wells × 100%. This procedure was repeated 6 times for each passage.

**Karyotype analysis**

The karyotype of P10 cells was analyzed as previously described (8). Cells were harvested when 80% to 90% confluent, subjected to hypotonic treatment, and fixed with the combination of glacial acetic acid and acetic acid. The chromosome numbers were counted from 100 spreads under an oil immersion objective after Giemsa staining.

**Flow cytometry analysis**

Cells were characterized by fluorescence-activated cell sorting (FACS) through cell surface markers. Umbilical cord MSCs of P7 were collected and then washed with PBS. Cells were resuspended in pre-cooling alcohol (70%) at 4°C overnight. After being washed by PBS and blocked by mouse serum, the sediment was stained with different monoclonal antibodies (BIOS). In brief, 1 × 10⁶ UCMSCs were harvested and fixed and incubated with antibodies to CD44, CD73, CD90, and CD105. After being washed with PBS, cells were incubated by FITC-conjugated goat anti-rabbit immunoglobulin. The fluorescence intensity was tested by EPICS-XL flow cytometry.

**Multi-potent differentiation of UCMSCs in vitro**

Passage 3 (P3) cells were sub-cultured on 6-well culture plates and complete medium was used until the cells reached 80% confluence. The medium was then changed to induced medium that contained 90% DF12, 10% FBS, dexamethasone (10 μmol/L), insulin (10 mg/L), indo-methacin (100 mM), and isobutylmethylxanthine (IBMX; 200 μmol/L) in 6-well plates at a density of 2 × 10⁵ cells/well. Medium was refreshed every 2 d. The undifferentiated control cells were fed with complete medium. After being cultured for 15 d and cell morphology in adipogenic induction medium observed, cells

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**Figure 3. Immunofluorescent staining: Characteristics of surface antigens of umbilical cord mesenchymal stem cells (UCMSCs). In-vitro cells were stained with antibodies to CD44, CD73, CD90, and CD105. Cells were counterstained with DAPI. (Scale bar = 50 μm).**
were stained with 0.3% Oil Red O (Sigma-Aldrich) for adipogenesis. The method was as follows: removing culture medium, washing the cells 3 times with PBS, fixing cells in 4% paraformaldehyde for 20 min, washing cells 3 times again with PBS, and adding the stain with 0.3% Oil Red O for 30 min. Finally, stained samples were examined on an inverted microscope.

**Chondrogenic differentiation of UCMSCs**

Passage 3 (P3) cells were subcultured on 6-well culture plates and complete medium was used until the cells reached 80% confluence. The medium was then changed to induced medium that contained 90% DF12, 10% FBS, 1% double antibody, a proline (40 mg/mL), dexamethasone (39 ng/mL), TGF-β3 (10 ng/mL), ascorbate 2-phosphate (50 mg/mL), sodium pyruvate (100 mg/mL), and insulin-transferrin-selenious acid mix (50 mg/mL) in 6 well-plates at a density of $2 \times 10^5$ cells/well. Medium was refreshed every 2 d. The undifferentiated control cells were fed with complete medium. After 21 d culture, the cells cultured in 2 kinds of culture medium were stained with Alcian Blue (Sigma-Aldrich). The method was as follows: removing culture medium, washing the cells with PBS 3 times, fixing cells in 4% paraformaldehyde for 20 min, washing cells with PBS 3 more times, and adding the stain with Alcian blue for 30 min. Finally, stained samples were examined on an inverted microscope.

**Results**

**Isolation, culture, and morphology of UCMSCs**

Primary cells were isolated from umbilical cord tissue and cultured on 6-well culture plates for 24 h (Figure 1). After being cultured for 6 d, cells reached approximately 80% to 90% confluence and expanded rapidly with a fibroblast-like morphology. There were no obvious morphological differences among different passages and cellular morphology remained stable after serial passages. When the primary cells isolated from umbilical tissue were cultured to P39, signs of cellular senescence began to show up for most of the cells. Vacuoles and karyopyknosis appeared if the confluence rate reached 70% to 80% in 7 d.

**Growth dynamics of UCMSCs**

The dynamic process of cell growth was similar with the proliferation of UCMSCs (Figure 2). Passage 4 (P4), P14, and P24 were shown as the growth curves, which were all typically sigmoidal. After a latent period of 3 d, cells reached a logarithm-increasing period. Cells then grew to a stable level and began to decrease after 7 d. The population-doubling times (PDTs) obtained were 8, 12, and 15 h for P4, P14, and P24, respectively.

**Immunofluorescence**

Markers of PSCs were detected by immunofluorescence staining and the results showed triple cells were positive with CD44, CD73, and CD105 (Figure 3).

**Reverse transcriptase-polymerase chain reaction (RT-PCR) assay**

The expression of 3 PSC genes by RT-PCR was appraised. The UCMSCs at different passages of CD29, CD44, and CD73 all showed positive expression, but did not express the CD34 gene. GAPDH was used as an internal control (Figure 4).
Colony-forming cells assay

Colony formation was observed under the microscope after 7 d (Figure 5). The colony-forming rates for passages 4, 14, and 24 were 59%, 50%, and 30%, all of which showed the ability of the cells to proliferate and self-renew in different passages.

Karyotype analysis

In this study, the sheep UCMSCs were diploid ($2n = 54$), containing 26 pairs of autosomes and 1 pair of sex chromosomes, which was XY(♂) type. The result showed genetic compatibility of UCMSCs cultured in vitro (Figure 6).

Flow cytometry analysis

Markers of passage 7 UCMSCs were analyzed by flow cytometry. The following genes were expressed: CD44, CD73, and CD105 and the positive rates were 99.98%, 99.73%, and 99.1%, respectively. The results confirmed that UCMSCs expressed specific stem cell surface markers CD44, CD73, and CD105 (Figure 7).

Multi-potent differentiation of UCMSCs in vitro

Umbilical cord MSCs of P3 were cultured in adipogenesis medium for 12 d, then UCMSCs turned into flat spindle and different sizes of fat droplets appeared in the center of cells. The fat droplets
increased in size 20 d after inducing (Figure 8). The results of RT-PCR showed that adipocyte-specific genes, such as LPL and PPAR-γ, were expressed in the cells being induced, but not in the control group.

**Osteogenic differentiation of UCMSCs**

Umbilical cord MSCs of P3 were cultured in osteogenic induction for 7 d, after which cell morphography noticeably changed in the cells. The cells changed from long fusiform to triangular in shape. Then the calcified nodules became larger and the number increased on day 12 (Figure 9). The results of RT-PCR showed that osteoblast-specific genes were expressed in the cells being induced, but not in the cells cultured in complete medium.

**Chondrogenic differentiation of UCMSCs**

On day 5 after UCMSCs of P3 were cultured in osteogenic induction, the cell confluence rate reached more than 90%. A dense cell layer appeared when cells continued to be cultured until day 14. These clusters formed 3D nodules with cells clustered into Brosette-like morphology (Figure 10). The cell clusters were then stained with Alcian blue. The result of RT-PCR showed that osteoblast-specific genes, such as gene Col-2 and Sox9, were expressed in the cells that were induced before, but not in the cells cultured in complete medium.

**Discussion**

For the past few years, mesenchymal stem cells have been regarded as the most promising cell resource for tissue repair and regeneration. However, the optimal source for clinical use has not been confirmed. Friedenstein and coworkers first discovered mesenchymal stem cells from bone marrow in 1966 (9). The quantity and ability of proliferation and differentiation of mesenchymal stem cells derived from bone marrow decreased with age and required a bone marrow biopsy for collection. Due to disease, the patients sometimes incurred an infection and became weak, which limited the application of auto bone marrow mesenchymal stem cells. The
use of embryonic stem cells was limited, however, by morality, ethics, and traditional concepts.

Umbilical cord mesenchymal stem cells were hard to passage and culture, which made them difficult to mass produce (10). As the immune cells of UCMSCs were less mature and their functional activity was also lower, however, they would not cause immune response and graft-versus-host disease (GvHD). At the same time, stem cells had high purity and were easily isolated. They are also not contaminated by tumor cells. It is easy to control in-cell proliferation because of the unified system for cell culture. Stems cells can be made into seed cells, the integrity of which is preserved after freezing and they can be used repeatedly. The risk of being infected by latent virus and pathogenic microorganisms is relatively low and their propagation is also slow. Stem cells have some advantages that make them an ideal seed cell (11,12). These advantages are: rich source, convenient method of collection and transportation, stable

Figure 9. Osteogenic differentiation of umbilical cord mesenchymal stem cells (UCMSCs). a — As a negative control, cells cultured in complete medium showed no changes in morphology. b — After induction in osteogenic medium for 7 d, the cells changed from long fusiform to triangular in shape. The calcified nodules became larger and the number increased on day 12. c — Alizarin Red staining. d — Reverse transcription polymerase chain reaction (RT-PCR) showed the expression of osteoblast-specific genes, including collagen I and osteopontin (OPN), in the induced group (2), but not in the control group (1).

Figure 10. Chondrogenic differentiation of umbilical cord mesenchymal stem cells (UCMSCs). a — Control cells. b — After induction in chondrogenic medium for 15 d, the induced cells changed from long fusiform to elliptic in shape and formed calcified nodules. c — Alcian blue staining. d — After induction for 15 d, reverse transcription polymerase chain reaction (RT-PCR) revealed the expressions of osteoblast-specific genes Sox9 and Col-1. (1) Sox9 and Col-2 were negative in the control group (1), but were positive in the induced group (2). GAPDH served as the internal control.
biological character, low or no expression of immune rejection genes, no allograft rejection, and no ethical issues. In this study, we focused on analyzing the biological characteristics of UCMSCs and, as a result, increased understanding of culturing UCMSCs in vitro.

Previous research has shown that umbilical cord mesenchymal stem cells (UCMSCs) are characterized by the capacity of self-renewing and unlimited proliferation and differentiation (13). In this study, we chose fetal lambs 3- to 4-months of age. Our samples were obtained from the farm operated by the Chinese Academy of Agricultural Sciences, which is situated close to our lab. The purity of small-tailed Han sheep was guaranteed. Mesenchymal stem cells were successfully isolated from umbilical cord and passaged to 29 generations. We observed and studied their cellular morphology and estimated the capacity of self-renewing and unlimited proliferation by growth curve and clonality.

We chose the following genes: CD29, CD44, CD73, CD90, and CD105 to test with RT-PCR and immunofluorescence. CD44 is a cell-surface glycoprotein involved in cell-cell interaction and migration and adhesion, which participates in various cellular functions including lymphocyte activation, recirculation, and homing. CD73 is a kind of glycoprotein that is distributed mainly at the cell surface and is anchored to plasma membranes by glycosylphosphatidylinositol (gpi). CD105 is a kind of glycoprotein that is related to cell proliferation and can induce hypoxia. The result showed a high positive expression of CD29, CD44, CD73, CD90, and CD105.

Differentiation potential is an important characteristic of stem cells. Stem cells can differentiate to cells of different layers by culturing with different inducing mediums. In this study, we induced cells from mesoderm to differentiate into osteogenesis cells, adipogenesis cells, and chondroblast cells. The expression level of these related genes from the 3 types of cells was detected by different staining assays and RT-PCR. Umbilical cord MSCs have a broad application potential because of their fine differentiative capacity. For example, chemotactic factors, such as chemokines SDF-1, which are secreted by tumor cells, and vascular endothelial cell growth factor, can simulate UCMSCs to migrate toward tumor cells, which makes them a tumor drug carrier in targeted therapy. In some studies, UCMSCs were cultured with brain tumor stem cells with the result that the co-culture limited the positive rate of surface marker CD133 from brain tumor stem cells. The co-culture also limited the proliferative capacity of cells and stimulated them to differentiate.

This study has shown that UCMSCs have a broad clinical application potential because of their immunological characteristics. The structure and functions of tissues and organs can be rebuilt through autotransplantation, which also avoids immunological rejection.

In conclusion, sheep umbilical cord stem cells (USMSCs) have strong self-renewal activity and can express surface makers well. They can differentiate toward multi-germinal layers. These results may lay a foundation for the use of UCMSCs from fetal sheep as a model of tendon pathological and repair mechanisms.

References

Isolation, culture, and characterization of chicken lung-derived mesenchymal stem cells

Xishuai Wang, FH C, JJ Wang, Hongda Ji, Weijun Guan, Yuhua Zhao

Abstract

Using lung tissues separated from 12-day-old chicken embryos, we attempted to obtain a novel population of stem cells, namely, chicken lung-derived mesenchymal stem cells (LMSCs), which exhibit spindle-like morphology.

The results of colony-forming assay and population doubling assay demonstrated that LMSCs had enormous colony-forming, self-renewal, and proliferative potential. When appropriately induced, LMSCs could differentiate into osteoblasts, adipocytes, chondrocytes, and neurons; in other words, LMSCs had cross-embryonic layer differentiation potential under corresponding induction conditions. Aside from colony-forming, self-renewal, and multilineage differentiation capabilities, LMSCs were characterized by specific cell phenotypes. The results of immunohistochemistry and flow cytometry demonstrated that LMSCs consistently expressed OCT-4 — a specific gene marker expressed in pluripotent stem cells — and markers associated with MSCs such as CD29, CD73, CD90, and CD105. However, LMSCs lacked hematopoietic cell surface molecules such as CD34 and CD45. Primary LMSCs could be subcultured to passage 24 at most in vitro and karyotype analysis demonstrated that LMSCs possessed genomic stability.

These unique characteristics were consistent with the characteristics of MSCs, which had been isolated from other tissues. This provides a foundation for LMSCs as a promising avenue for cellular transplantation therapy, regenerative medicine, and tissue engineering.

Introduction

Mesenchymal stem cells (MSCs) were originally separated from bone marrow (1). Similar populations of MSCs have been identified in virtually all tissues such as pancreas, skin, kidney, heart, muscle, umbilical cord, and amnion (2–8). In addition, several subsets of MSCs have been isolated from lung tissue from humans, rabbits, cattle, and sheep (9–12). At present, there are no specific MSC phenotypes; MSC expression is generally confirmed by previously identified phenotypes (13–14). Using a combination of morphological characteristics, self-renewal capabilities, proliferative potential, multiple differentiation potential, and certain phenotypes, MSCs can be identified (15).

Mesenchymal stem cells offer practical advantages including ease of separation, culture, and proliferation. Moreover, MSCs come from a wealth of sources and can be harvested at a low cost.

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Drs. Xishuai Wang and C contributed equally to this work.
Compared with embryonic stem cells, MSCs have a limited risk of tumor formation and do not pose ethical dilemmas (16). These cells possess low immunogenicity and potent immunomodulatory and anti-inflammatory properties in several autoimmune and inflammatory diseases. Namely, MSCs interact with several major types of innate and adaptive immune cells such as T-lymphocytes, B-lymphocytes, NK cells, and dendritic cells, and modulate the inflammatory reaction (16–18). Besides, the protective effects of MSCs in chronic degenerative diseases, and effects on injuries in the lung, liver, kidney, spinal cord, muscle, and tendon, have been demonstrated (19–26). Recently the underlying mechanisms of MSC therapy were clarified. Accumulating evidence has revealed that MSCs protect against several diseases through various mechanisms such as engraftment, immunomodulation, anti-oxidative stress, and anti-fibrosis (27–30). However, existing studies have mainly focused on human, rabbit, and mouse models; few experiments have been performed on chickens (30–34). Compared to humans, rabbits, and mice, chicken embryos are more readily available. This makes the study of MSCs more economic, which is vital for the promotion of stem cell research.

### Isolation, culture, purification, and cryopreservation of lung-derived mesenchymal stem cells

The lung tissues were separated from the chicken embryos under sterile conditions and then washed 5 to 7 times using sterile phosphate-buffered saline (PBS) to remove as many blood cells as possible. After washing, arteries, veins, connective tissues, and the outer layer of lung tissues were carefully removed. The remaining tissues were mechanically minced into small fragments (1 mm³) and dissociated with 0.125% trypsin/EDTA and then subcultured at a ratio of 1:2.

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When LMSCs reached 90% confluence, they were digested and harvested in 15 mL sterile centrifuge tubes, as previously described, and cell pellets were resuspended with frozen stock solution (50%...
FBS, 40% DMEM/F12, and 10% DMSO). A 1-mL volume of the resulting suspension (1 × 10⁶ cells/mL) was transferred to a 1.8 mL sterile frozen tube, which was followed by overnight refrigeration at −80°C. The LMSCs were transferred into liquid nitrogen for long-term preservation.

**Population doubling assay**

Lung-derived MSCs at 3 different passages (P3, P13, and P23) were digested and plated into a 96-well plate at a density of 1 × 10⁴ cells/well. The culture expanded for 7 d and the average number of cells in the 10 dishes was used to depict the curves of proliferation. The population doubling time (PDT) was calculated as follows:

\[ \text{PDT} = (t - t_0) \frac{\log 2}{\log \left( \frac{N}{N_0} \right)} \]  

*(Equation 1)*

Where: \( N \) and \( N_0 \) represent the cell numbers at time \( t \) and \( t_0 \), respectively.

**Single cell cloning**

Lung-derived MSCs of passages 3, 13, and 23 were digested using 0.125% trysin/EDTA. A serial dilution was used to produce single-cell suspension and then 100 μL of the final diluted cell suspension (1000 cells/mL) was seeded onto a 60-mm culture plate. On culture day 14, LMSCs were stained with Giemsa and colony forming efficiency (CFE) was calculated using the following formula:

\[ \text{CFE} = \frac{\text{colony-forming unit number}}{\text{starting cell number}} \times 100\% \]  

*(Equation 2)*

**Karyotype analysis**

When MSCs reached 80% to 90% confluence, they were subjected to hypotonic treatment, fixed, and stained according to the previously described G-banding protocol (35). After staining, the number of chromosome bands was counted, and the results were recorded.
Figure 2. Self-renewal and proliferative potential of lung-derived mesenchymal stem cells (LMSCs). A — Colony-forming cell assay. On culture day 7, colony-forming units of P3, P13, and P23 of chicken LMSCs were counted and indicated that colony-forming efficiency decreased with the passaging. (a), (b), and (c) Colony-forming rates of P3, P13, and P23, respectively. (d) Bar chart of colony-forming efficiency for different passages of chicken LMSCs whose colony-forming efficiency rates were 81.5%, 63.6%, and 42.5% at passages P3, P13, and P23, respectively. The colony forming efficiency of LMSCs at P3 was significantly higher than at P13 and P23 (*P < 0.01) and P13 was significantly higher than P23 (**P < 0.01; Figure 3D). ** — Compared to P3 (*P < 0.01); ## — Compared to P13 (**P < 0.01). B — Growth curves of chicken LMSCs. The growth curves from passages P3, P13, and P23 were all typically sigmoidal in shape, with cell density reflected by the vertical axis. The growth curve consisted of a lag phase, a logarithmic phase, and a plateau phase.
of chromosomes was calculated for metaphase spreads. The morphology of chromosomes was observed and images were captured under an oil immersion objective.

**Immunocytochemistry**

When LMSCs proliferated to 40% to 50% confluence, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, followed by a 3 x 5 min wash. A 0.1% Triton X-100 solution was used to permeabilize LMSCs for 30 min, followed by a 3 x 5 min wash. Non-specific binding sites were blocked using PBS containing 10% sheep serum for 30 min at room temperature, followed by 3 x 5 min wash. After blocking FITC-rabbit OCT-4, CD29, CD73, CD90, CD105, CD45 antibodies were incubated overnight at 4°C, followed by 3 x 5 min wash. The supernate was discarded and the cells were incubated in PBS containing FITC-goat anti-rabbit secondary antibody for 1 h in the dark, at room temperature. After incubation, LMSCs were washed 3 times with PBS (5 min each) and counterstained with 4',6-diamidino-2-phenylindole for 10 min at room temperature, in the dark. Eventually, fluorescent signals were detected by a fluorescence microscope.

**Adipogenic differentiation of lung-derived mesenchymal stem cells**

Third-generation LMSCs were inoculated at 2.0 x 10^4 cells/well in 12-well plates. When cells were 90% confluent, they were placed on a medium designed to induce adipogenic differentiation, composed of DMEM/F12, 10% FBS, 1 mM dexamethasone, 0.5 mM IBMX, 10 ng/mL insulin, and 60 mM indomethacin. The adipogenic differentiation medium was refreshed with 50% fresh medium every other day for a period of 21 d. Afterward, the accumulated intracellular lipid droplets were visualized by Oil Red O staining and the adipogenic marker genes — peroxisome proliferator-activated receptor-γ (PPAR-γ) and lipoprotein lipase (LPL) — were detected by reverse transcription polymerase chain reaction (RT-PCR).

**Osteogenic differentiation of lung-derived mesenchymal stem cells**

When LMSCs were approximately 80% to 90% confluent, the medium was replaced with osteogenic medium supplemented with DMEM/F12 medium, 10% FBS, 10 nmol/L dexamethasone, 10 nmol/L β-glycerophosphate, and 50 mg/L ascorbic acid. The osteogenic medium was refreshed with 50% fresh medium every 2 d for a period of 21 d. Afterward, calcium deposition was visualized using Alizarin Red S staining and osteogenic specific genes [osteopontin (OPN) and collagen type 1 (Col-1)] were identified by RT-PCR.

**Chondrogenic differentiation of lung-derived mesenchymal stem cells**

For chondrogenic differentiation, LMSCs were cultured in chondrogenic medium composed of 10% FBS, 1% ITS, 50 μg/mL L-proline, 50 μg/mL vitamin C, 0.9 mM sodium pyruvate, 0.1 μM dexamethasone, and 10 ng/mL TGF-β3. Chondrogenic medium was refreshed with 50% fresh medium every 2 d. On culture day 21, Alcian blue staining was performed to confirm chondrogenic differentiation. Eventually, chondrogenic specific genes Sox9 and Col-2 were further assayed by RT-PCR.

**Figure 3. Karyotype analysis of lung-derived mesenchymal stem cells (LMSCs).** The chromosomes of chickens were normal diploid (2n = 78), consisting of 9 pairs of macrochromosomes, a pair of sex chromosomes, and 30 pairs of microchromosomes. The sex chromosome type was ZZ. No chromosome deletions or mutations appeared.
Neuroblastic differentiation of lung-derived mesenchymal stem cells

When 80% to 90% confluence was achieved, LMSCs were replaced with DMEM/F12 supplemented with 5% FBS, 2% B27, 2 mM L-glutamine, 40 ng/mL bFGF, 20 ng/mL EGF 10 ng/mL glial cell-derived neurotrophic factor, and 50 μg/mL ascorbic acid. The medium was replaced with 50% fresh medium every 2 d. On day 21 of differentiation, neural markers were identified by immunohistochemical staining and morphological changes were captured under inverted microscope.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

TRIzol reagent (Invitrogen) was used to extract total RNA from LMSCs using the previously described method (12). Total RNA (2.0 μg) was reverse transcribed into cDNA using a RNA PCR Kit (AMV) Version 3.0 (Takara). An optical density ratio of 260/280 was used to identify RNA concentration and purity. The cDNA was denatured at 94°C for 5 min and RT-PCR continued for 35 cycles including 30 s at 94°C, 30 s at annealing temperature, 30 s at 72°C, and 10 min at 72°C for cDNA extension. All primers are listed in Table I. The PCR products of differentiated and undifferentiated LMSCs were visualized by 2% agarose gel electrophoresis using ethidium bromide as a fluorescent tag. Undifferentiated LMSCs were used as negative control and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the reference gene.

Flow cytometry

The LMSCs were dissociated into a single cell suspension. After counting, 1 × 10⁶ cells were transferred into a 15 mL sterile centrifuge tube and centrifuged at 1200 rpm for 8 min. After centrifugation, the supernatant was discarded and cells were washed twice with PBS. The precooled 70% ethanol was added to the centrifuge tube dropwise in order to fix LMSCs, which were then incubated overnight at 4°C and centrifuged at 1200 rpm for 8 min at room temperature. The cells were incubated overnight at 4°C in PBS containing the following primary antibodies raised in rabbit: anti-CD29, anti-CD73, anti-CD90, anti-CD105, anti-OCT-4, anti-CD34, and anti-CD45. This was followed by a 3 × 5 min wash. Cells were incubated
in PBS containing FITC-labeled goat anti-rabbit IgG in the dark for
60 min at room temperature, followed by a 3 × 5 min wash. Finally,
expression of cell surface markers was detected by flow cytometry.

## Results

### Morphological observation of lung-derived mesenchymal stem cells

In this study, we successfully separated single-colony derived
MSCs from lung tissue of chicken embryos. The primary cells, which
were initially round or irregularly shaped, began to stick to the
well after 24 h in culture. At the early stage of cell culture, the cells
were heterogeneous because endothelial cells and hemocytes were
concomitant in the primary culture. After 2 to 3 passages, all cells
except for LMSCs detached from the population and were eliminated
gradually. Morphological differences disappeared and cell morphol-
omy became consistent (Figure 1A-a). Typically, primary cells reached
80% to 90% confluence after 5 to 7 d in culture (Figure 1A-b) and
then rapidly proliferating LMSCs could be subcultured every 24 to
48 h (Figure 1A-c). Lung-derived MSCs could be cultured for up to
25 passages in vitro. By the 20th generation of subculture, LMSCs
appeared to be aging and cells began to display signs of senescence,
such as slow proliferation (Figure 1A-d). As the passage number
increased, LMSCs detached gradually from the plates.

### Detection of lung-derived mesenchymal stem cells

The results of immunocytochemistry demonstrated that ~100%
of LMSCs were positive for CD29, CD73, CD90, CD105, and OCT-4,
whereas the expression of CD34 and CD45 was negative (Figure 1B).
These findings were consistent with the results of flow cytometry
(Figure 1C).

### Colony-forming ability

On culture day 7, CFE of LMSCs were 81.5%, 63.6%, and 42.5%
at passages P3, P13, and P23, respectively, implying the enormous
capacity of cultured LMSCs for self-renewal (Figure 2Aa-c). The
CFE was significantly higher at P3 than P13 (P < 0.01) and CFE was
significantly higher at P13 than P23 (P < 0.01; Figure 2A-d).

### Self-renewal and proliferative capabilities of lung-derived mesenchymal stem cells

Lung-derived MSCs have active proliferative capacity and the
growth curves typically appear sigmoidal. The growth curves are
composed of lag, logarithmic, and plateau phases. After a lag phase
of 2 d, LMSCs grew rapidly and entered the logarithmic phase and then plateaued around day 7. The biological analysis results showed that the PDT of LMSCs from 3 different passages (P3, P13, and P23) were approximately 24.28 h, 34.86 h, and 46.25 h, respectively. The proliferation capability of LMSCs declined as the passage number increased (Figure 2B).

**Karyotype analysis**

Chicken chromosomes were normal diploid \( (2n = 78) \), consisting of 9 pairs of macrochromosomes, a pair of sex chromosomes, and 30 pairs of microchromosomes. No chromosome deletion or mutation occurred. Chromosomes’ structural integrity demonstrated that cultured LMSCs possessed hereditary stability (Figure 3).

**Adipogenic differentiation of lung-derived mesenchymal stem cells**

Evident morphological alternations were observed with the induction time extended to 21 d. Unlike the control group (Figure 4A), the experimental group had lipid droplets in the cytoplasm. Intracellular lipid droplets were visualized by positive Oil Red O staining (Figures 4B and 4C). The RT-PCR results revealed that the expression of adipocyte-specific genes, including PPAR-\( \gamma \) and LPL, was positive in differentiated LMSCs, while the expression of PPAR-\( \gamma \) and LPL was negative in undifferentiated LMSCs (Figure 4D).

**Osteogenic differentiation of lung-derived mesenchymal stem cells**

Compared to the control cells (Figure 5A), experimental LMSCs cultured in osteogenic medium for 21 d had calcium nodules in the cytoplasm and accumulated calcium crystals stained positive with Alizarin Red S (Figures 5B and 5C). The RT-PCR results show that LMSCs highly expressed osteoblast-specific genes OPN and Col-1 in differentiated cells, while those genes could not be detected in undifferentiated cells (Figure 5D).

**Chondrogenic differentiation of lung-derived mesenchymal stem cells**

Compared to the control group (Figure 6A), LMSCs were successfully stained with Alcian blue after incubation in chondrogenic medium for 21 d (Figures 6B and 6C). The RT-PCR results show that
specific genes including Col-2 and Sox9 were detected in differentiated LMSCs (Figure 6D) while those genes could not be detected in undifferentiated cells (Figure 7B-b). The results reveal that LMSCs could differentiate into neurons when appropriately induced.

**Discussion**

In this study, a novel subset of stem cells was successfully separated from chicken lung tissue and an efficient system was established for isolation and culture of LMSCs. More importantly, LMSCs were characterized by their morphological characteristics, clonogenicity, self-renewal, proliferative, and multi-directional differentiation capabilities, and specific surface markers.

The LMSCs cultured *in vitro* attained at least 23 serial passages, implying that chicken LMSCs can provide a rich source of cells. More significantly, LMSCs are convenient to isolate, culture, and proliferate. Sources of chicken embryos are abundant, which make them an economic choice. Hence, this study would contribute to existing literature on the applications of MSCs.

It has been well-documented that MSCs possess enormous self-renewal and proliferative capabilities (37–38). Similarly, colony-forming assays and population doubling assays demonstrated that LMSCs also had those characteristics. The results of colony-forming assays revealed that as the passage number increased, CFE of LMSCs decreased significantly. In other words, LMSCs have potent but limited colony-forming capability, which declined in a passage-dependent manner. The LMSCs, therefore, have a limited risk of tumor formation and the results confirmed the results of previous experiments (13,24).

Lung-derived MSCs exhibited sigmoidal growth curves; namely, they went through 3 phases: latent, logarithmic, and plateau (39). At first, LMSCs had an extremely slow growth speed owing to low density and recovery of damage caused by enzymes. After a 2 to 3 d latency phase, LMSCs proliferated rapidly and entered the logarithmic phase and then plateaued as a result of increased cell density and contact inhibition (40). As the passage number increased, PDT prolonged significantly. The results demonstrate that the proliferative capability of LMSCs declined significantly. They also indicate that MSCs have potent but limited self-renewal and proliferative capabilities. The results are consistent with previous research (40–43).

Aside from self-renewal and proliferative ability, LMSCs have great heterogeneity in their differentiation potential *in vitro* (44–47). Chicken lung-derived MSCs, which originate from mesoderm lineage, could be induced to differentiate into cells with a mesoblastic origin (e.g., adipocytes, osteoblasts, and chondrocytes) or an ectoderm origin (e.g., neurons), namely, LMSCs with cross-embryonic layer differentiation potential. The results demonstrate that the direction of differentiation of LMSCs was determined by different inducing mediators. However, underlying differentiation mechanisms of MSCs remain to be elucidated. Hence, further research into inducing mediators and differentiation mechanisms is desperately needed due to its importance for cell-based treatment and regenerative medicine.

In conclusion, using lung tissue separated from 12-day-old fertilized eggs, we obtained a new population of stem cells, which possessed MSC-associated properties such as a fibroblast-like morphology, clonogenicity, self-renewal, proliferative, and multi-directional differentiation capabilities. These properties offer a
scientific foundation for chicken LMSCs and make them a promising alternative for cellular transplantation therapy.

Acknowledgments

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References


ACTN3 gene variants as potential phenotype and performance biomarkers in Brazilian sport horses training for eventing in a tropical climate

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Abstract

The aim of this study was to look for mutations in the equine ACTN3 gene and to identify sequence variants that might be associated with the phenotype and performance of Brazilian sport horses training for events in a tropical climate. Among 17 such horses direct DNA sequencing and mutation analysis of the exon 15 and the intron–exon boundaries of ACTN3 revealed 2 new sequence variants in the ACTN3 intron 14–15, designated c.1681–86G > A and c.1681–129delA. Wild-type/deletion heterozygotes (A/del) had a lower mean subcutaneous fat layer in the region of the gluteus medius, as measured by ultrasonography, than the del/del homozygotes; the correlation was significant (P = 0.017). This single base-pair deletion in ACTN3 intron 14–15 may have resulted in metabolic changes that led to increased deposition of body fat in the homozygous state. However, neither sequence variant was correlated with the time to fatigue in a test on a high-speed treadmill with an incremental-speed protocol.

Résumé

Le but de la présente étude était de vérifier la présence de mutations dans le gène ACTN3 équin et d’identifier des variants de séquence qui pourraient être associés avec le phénotype et la performance de chevaux de sport brésiliens qui s’entraînent pour des concours dans un climat tropical. Parmi 17 chevaux qui correspondent à ces critères, le séquençage direct de l’ADN et l’analyse de mutation de l’exon 15 et des frontières de l’intron-exon d’ACTN3 a révélé deux nouveaux variants de séquence dans l’intron 14–15 d’ACTN3, désigné c.1681–86G > A et c.1681–129delA. Chez les hétérozygotes type-sauvage/déletion (A/del) la moyenne de l’épaisseur de la couche de gras sous-cutanée dans la région du glutteus medius était plus petite, telle que mesurée par échographie, que celle des homozygotes del/del; la corrélation était significative (P = 0.017). Cette délétion unique de paire de bases dans l’intron 14–15 d’ACTN3 pourrait avoir réduit des changements métaboliques qui auraient mené à une augmentation du dépôt de gras chez les homozygotes. Toutefois, aucun des variants de séquence n’était corrélé avec le temps de fatigue dans un test sur un tapis-roulant à haute vitesse avec un protocole d’augmentation de vitesse.

Alpha-actinin-3 (ACTN3) is an actin-binding protein specific to fast-twitch muscle fibers that influences power generation in high-speed activities (1). A deficiency of ACTN3 results in a shift in muscle metabolism from the glycolytic pathway toward the oxidative pathway (2–5). Low ACTN3 expression is related to a reduction in the diameter of fast-twitch fibers, increased activity of aerobic enzymes, a change in contractile properties, and improved recovery from fatigue (1,2,4,6). The aim of this study was to look for mutations in the exon 15 and the intron–exon boundaries of equine ACTN3 and to identify sequence variants that might be associated with phenotype and performance of Brazilian sport horses training for events in a tropical climate. The study procedures were approved by the Ethics Committee for Use of Animals of the Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil (protocol 276/2013).

Of the 17 Brazilian sport horses in training for such events that were selected for genotyping, 6 were mares and 11 were geldings. Their ages ranged from 4 to 10 y, with a mean of 7.59 ± 1.87 [standard deviation (SD)] y. They were housed in masonry stalls 4 × 4 m with free access to water, and 3 times a day they were fed “coast-cross” hay (Cynodon dactylon L. Pers.) and commercial concentrate (1% body weight) containing guaranteed levels of the following:

- maximums for:
  - fibrous matter — 150 g/kg (15%);
  - acid detergent fiber — 180 g/kg (18%);
  - mineral matter — 120 g/kg (12%); and
  - calcium — 20 g/kg (2%).
- minimums for:
  - crude protein — 120 g/kg (12%).
ethereal extract — 40 g/kg (4%);
calium — 15 g/kg (1.5%);
phosphorus — 5000 mg/kg (0.5%);
digestible energy — 2700 kcal/kg;
methionine — 1800 mg/kg;
lysine — 4800 mg/kg;
vitamin A — IU/kg;
vitamin D3 — 1000 IU/kg;
vitamin E — 100 IU/kg;
vitamin B1 — 5 mg/kg;
vitamin B2 — 4 mg/kg;
copper — 20 mg/kg;
iodine — 0.5 mg/kg;
manganese — 64 mg/kg;
selenium — 0.2 mg/kg;
cobalt — 0.14 mg/kg; and
zinc — 80 mg/kg.

During the year of the study the average minimum and maximum temperatures in Rio de Janeiro were 21.7°C and 30.3°C according to the Brazilian National Institute of Meteorology; the mean relative humidity was 72.67% (7).

Mutation analysis of the ACTN3 gene regions of interest was by direct DNA sequencing, according to Mata et al (8). The equine ACTN3 reference genomic sequence used in the analysis was retrieved from GenBank [National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA] with the accession number NC_00009155.2.

The thickness of the subcutaneous fat layer was measured in the region of the equine muscle gluteus medius by ultrasonography. Effort was determined with an incremental speed test on a high-speed treadmill, the horses exercising at 8.0 m/s until fatigued (Table I). The treadmill inclination simulated the rider’s weight.

The significance of associations between quantitative variables was determined with Pearson’s correlation coefficient and significance testing. Differences in qualitative variables between independent groups were compared by means of Fisher’s exact test. Between-group differences in the value of quantitative variables were compared by means of the nonparametric Mann–Whitney test. A 5% significance level was used in all tests. Statistical analysis was done with the use of SPSS software, Version 17 (Unicom, Mission Hills, California, USA).

Table 1. Protocol of an incremental speed test on a high-speed treadmill for 17 Brazilian sport horses training for events in a tropical climate.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time (min)</th>
<th>Velocity (m/s)</th>
<th>Gait</th>
<th>Incline (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm-up</td>
<td>2</td>
<td>1.7</td>
<td>Walk</td>
<td>0</td>
</tr>
<tr>
<td>Warm-up</td>
<td>4</td>
<td>4.0</td>
<td>Trot</td>
<td>0</td>
</tr>
<tr>
<td>Warm-up</td>
<td>4</td>
<td>4.0</td>
<td>Trot</td>
<td>3</td>
</tr>
<tr>
<td>Gallop</td>
<td>1</td>
<td>5.0</td>
<td>Gallop</td>
<td>3</td>
</tr>
<tr>
<td>Gallop</td>
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<td>Gallop</td>
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<td>3</td>
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<td>Gallop</td>
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<td>8.0</td>
<td>Gallop</td>
<td>3</td>
</tr>
<tr>
<td>Recovery</td>
<td>10</td>
<td>1.7</td>
<td>Walk</td>
<td>0</td>
</tr>
</tbody>
</table>

Adapted from Couroucé-Malblanc and Hodgson (9).

Figure 1. Significant correlation ($P = 0.017$) between the mean thickness ($\pm$ standard deviation) of the subcutaneous fat layer in the region of the gluteus medius, as measured by ultrasonography, and the ACTN3 sequence variant c.1681–129delA in 17 Brazilian sport horses training for events in a tropical climate.

Demonstrating that the analyzed genotypes were randomly sampled from the general population.

Neither the c.1681–129delA variant nor the c.1681–129delA variant of ACTN3 intron 14–15 had previously been described, and these variants are distinct from the ACTN3 haplotypes reported in horses by Mata et al (8) and Thomas et al (10).

The mean thickness ($\pm$ SD) of the subcutaneous fat layer in the region of the gluteus medius was 0.464 ± 0.242 cm. Genotype and phenotype comparison revealed a significant correlation ($P = 0.017$) of the sequence variant c.1681–129delA with the thickness of the subcutaneous fat layer: the heterozygous A/del horses had a lower body fat thickness with less variability compared with the del/del homozygotes (Figure 1). The horses maintained a speed of 8.0 m/s on the high-speed treadmill until becoming fatigued for 127 to 386 s, with a mean $\pm$ SD of 280.24 ± 74.52 s. Neither sequence variant was correlated with the time to fatigue.
An investigation in ACTN3 mutant mice found a correlation between decreased weight gain and decreased size of the subcutaneous fat depot in individuals with predominantly slow type I muscle fibers (11). Thus, a switch in muscle metabolism could account for the findings in our study among the heterozygous horses with the variant c.1681–129delA. The phenotype of the horses training for events in a tropical climate may have influenced the selection of horses with these polymorphisms for our study. Brazil’s hot and humid environment and the type of equestrian sport may have led to more adaptation of sports horses, as those with less subcutaneous fat would be more suited for an event in a tropical climate.

In conclusion, the c.1681–129delA mutation in the ACTN3 intron 14–15, when homozygous, may have resulted in metabolic changes that led to increased deposition of body fat in Brazilian sport horses training for events in a tropical climate. This intronic single base-pair deletion should be considered for additional study in Brazilian sport horses and other equine breeds to evaluate its potential as a biomarker of phenotype and physical performance.

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References

Effect of piglet separation from dam at birth on colostrum uptake
Rodrigo Manjarín, Yanisse A. Montano, Roy N. Kirkwood, Darin C. Bennet, Kiro R. Petrovski

Abstract
The objective of this study was to determine whether birth order influences piglet survival because of reduced uptake of maternal antibodies by the piglets born later in large litters. Forty-five litters were serially allocated to one of 2 study groups. The crèche group consisted of 18 litters for which the 205 piglets were removed to a warm box to prevent suckling until 4 h after delivery of the first pig and the control group of 27 litters for which 306 piglets were allowed to suckle from birth. The protein content of piglet blood and sow colostrum was determined with Brix refractometers. Parity, farrowing duration, liveborn litter size, litter size at 12 d, and piglet weight at birth and at 24 h and 12 d of age did not differ between the 2 treatment groups (P > 0.1). There were also no significant differences (P > 0.1) at any time point in weight or in the mean percent protein in plasma between the first 3 and the last 3 piglets born to an individual sow. However, the mean percent protein in plasma was significantly higher in the control group than in the crèche group at both 24 h (P ≤ 0.05) and day 12 (P ≤ 0.01) postpartum. The lack of differences in plasma protein levels between the first and last pigs born along with the lower percent plasma protein in the piglets that were prevented from sucking immediately after birth militate against the use of this technique as a way to equalize the opportunity for adequate transfer of maternal antibodies.

Résumé
L’objectif de la présente étude était de déterminer si l’ordre de naissance influence la survie des porcelets à cause de la réduction d’ingestion d’anticorps maternels par les porcelets nés plus tard dans les portées nombreuses. Quarante-cinq portées ont été réparties de manière successive à l’un des deux groupes d’étude. Le groupe crèche était constitué de 18 portées pour lesquelles les 205 porcelets ont été maintenus dans une boîte chauffée pour empêcher la tétée jusqu’à 4 h après la naissance du premier porcelet, et le groupe témoin de 27 portées pour lesquelles les 306 porcelets ont pu têter dès la naissance. Le contenu en protéine du sang des porcelets et du colostrum de la truie a été déterminé avec un réfractomètre Brix. La parité, la durée de la mise-bas, le nombre de porcelets nés vivants, la taille de la portée à 12 j, et le poids des porcelets à la naissance et à 24 h et 12 j d’âge n’étaient pas différents entre les deux groupes de traitement (P > 0,1). Il n’y avait également pas de différence significative (P > 0,1) à aucun des temps mesurés pour le poids ou le pourcentage moyen de protéines dans le plasma entre les trois premiers et les trois derniers porcelets nés à une truie individuelle. Toutefois, le pourcentage moyen en protéine dans le plasma était significativement plus élevé dans le groupe témoin que dans le groupe crèche à 24 h (P ≤ 0,05) et au jour 12 (P ≤ 0,01) post-partum. Le manque de différence dans les quantités de protéines plasmatiques entre le premier et le dernier né avec également le pourcentage de protéine plasmatique plus faible chez les porcelets qui ont été empêché de boire immédiatement après la naissance militait contre l’utilisation de cette technique comme moyen d’égaliser l’opportunité pour un transfert adéquat d’anticorps maternels.

Genetic selection for high prolificity among sows over the past decades has resulted in increased litter size but also lower piglet survival (1). Nearly 80% of deaths occur during parturition and within the first 3 to 4 d of life (2). Pigs are born with an immature active immune system and, owing to intrauterine placental barriers, without maternal antibodies. Ingestion of colostrum soon after birth is essential to systemic immunologic protection via absorption of maternal antibodies before gut epithelial closure (3). Factors affecting early nursing among piglets, such as birth weight and birth order, have been correlated with both blood immunoglobulin (Ig) levels (4,5) and survival rates (6). Decreased Ig levels in late-born piglets have been attributed to a rapid decline in the Ig content of sow colostrum between the times that the first and last piglets are born (7–9). Since protein and Ig fractions in colostrum were found to be maintained when sucking was prevented (8), several authors have recommended removal of the litter at birth to confer on all piglets an equal opportunity for adequate transfer of maternal antibodies (7,8,10). However, split sucking (11) and nursing restriction immediately after farrowing (12) have yielded inconsistent results. Differences in blood Ig levels between early-born and late-born piglets after litter removal and subsequent replacement therefore required further investigation.

Several assays are available for evaluating the transfer of passive immunity from the dam to the piglets. Radial immunodiffusion provides direct measurement of the IgG concentration in serum and colostrum, but, although this test is the industry gold standard, it is...
resulted in relative slow and expensive. Conversely, digital Brix refractometers provide fast and reliable estimations of the IgG concentration in both colostrum and serum (13,14). These devices measure the percentage of sucrose in liquids, and when used in non-sucrose-containing liquids the readings approximate the percentage of total solids (15), which in sow colostrum and neonatal serum is mostly IgG (16).

We hypothesized that placing newborn piglets in a warm box (crèche) to prevent suckling for 4 h and then replacing all the piglets with the dam at the same time would increase the percentage of protein in the plasma of the last-born piglets compared with allowing the entire litter immediate access to the dam. The study was carried out at the University of Adelaide piggery in Roseworthy, South Australia, and was approved by the local animal ethics committee.

Forty-five litters born over 2-week-long periods to mixed-parity sows (Large White x Landrace) were serially allocated to 1 of 2 treatment groups at farrowing. In the crèche group the piglets were placed in a warm box in the farrowing pen to prevent suckling until 4 h after delivery of the first pig (18 litters, 205 piglets). In the control group the piglets stayed with their dam and were permitted to nurse immediately after birth; additional control litters became available and were included in the study, for a total of 27 litters and 306 piglets. Individual piglet weights were recorded at birth, at 24 h, and on day 12 of lactation. Additionally, records of birth order, size of liveborn litter at birth and at 12 d, and farrowing duration were available. Cross-fostering was not permitted before 24 h.

Blood samples (3 mL) were collected by jugular venipuncture at 24 h and 12 d after farrowing from the first 3 and the last 3 piglets born in each litter. Plasma was harvested after centrifugation (for 24 h and 12 d after farrowing from the first 3 and the last 3 piglets available. Cross-fostering was not permitted before 24 h.

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Forty-five litters born over 2-week-long periods to mixed-parity sows (Large White x Landrace) were serially allocated to 1 of 2 treatment groups at farrowing. In the crèche group the piglets were placed in a warm box in the farrowing pen to prevent suckling until 4 h after delivery of the first pig (18 litters, 205 piglets). In the control group the piglets stayed with their dam and were permitted to nurse immediately after birth; additional control litters became available and were included in the study, for a total of 27 litters and 306 piglets. Individual piglet weights were recorded at birth, at 24 h, and on day 12 of lactation. Additionally, records of birth order, size of liveborn litter at birth and at 12 d, and farrowing duration were available. Cross-fostering was not permitted before 24 h.

Blood samples (3 mL) were collected by jugular venipuncture at 24 h and 12 d after farrowing from the first 3 and the last 3 piglets born in each litter. Plasma was harvested after centrifugation (for 10 min at 1300 x g) and stored at -20°C until assayed for protein content. Colostrum (5 mL) was manually collected from the anterior, middle, and rear glands immediately after the start of farrowing and refrigerated until the protein assays were done, within 4 h.

Indirect measurements of the IgG content of plasma and colostrum were obtained in duplicate with commercial digital hand-held refractometers (Atago PAL-115 for plasma and STARR DBR-1 for colostrum; STARR Instruments, Dandenong South, Victoria, Australia). Approximately 50 μL of plasma or colostrum was placed on the refractometer and the Brix percentage recorded. Approximately 40 samples each of plasma and colostrum were also assayed by the University of Adelaide Veterinary Diagnostic Laboratory to validate the results obtained by Brix refractometry. A correlation of 98.8% was obtained between the Brix values and the measured total protein percentage in the samples (data not shown); henceforth, the refractometry data will be referred to as percent protein.

Percent protein in plasma and colostrum as well as gain in piglet weight from birth were assessed by analysis of variance with the use of linear mixed models (PROC MIXED; SAS Institute, Cary, North Carolina, USA) that included treatment group, piglet birth order, time (birth, 24 h, or 7 d), and mammary gland position, as well as their interactions as fixed effects, litter and parity as random effects, and piglet weight and litter size as covariates. If an interaction was found not to be significant it was removed from the model and the data were reanalyzed. Normality of the residuals and the presence of outliers were assessed by PROC UNIVARIATE (SAS Institute) with use of the Shapiro–Wilk test, quantile–quantile plots (for determining if 2 data sets come from populations with a common distribution), and externally studentized residuals (deleted residuals divided by their estimated standard deviation). Non-normally distributed parameters were power-transformed by the parameter r, whose optimal value was estimated by the maximum likelihood method. Pairwise comparisons were analyzed by Student’s t-tests. Data are presented as least-squares means ± the standard error. Significance effects were considered to have a P-value of 0.05 or less. Correlations were determined with use of the PROC CORR procedure (SAS Institute). Pearson’s correlation coefficient was considered significant at a P-value of 0.05 or less.

Results are shown in Table I. Mean parity, farrowing duration, number of piglets born alive, number of piglets alive on day 12, percent protein in the sow colostrum, and piglet body weight at birth and at 24 h and 12 d of age did not differ significantly between the 2 treatment groups (P > 0.1). There were also no significant differences (P > 0.1) at any time point in weight or in the mean percent protein in plasma between the first 3 and the last 3 piglets born to an individual sow. However, the mean percent protein in plasma was significantly higher in the control group than in the crèche group at both 24 h (P ≤ 0.05) and 12 d (P ≤ 0.01) postpartum. The percent protein in colostrum was not affected (P > 0.1) by gland position (data not shown). There was a positive relationship for percent protein in the plasma of the piglets between 24 h and 12 d of age (r = 0.44; P ≤ 0.0001). Likewise, piglet weight at 24 h was related to piglet weight at 12 d postpartum (r = 0.76; P ≤ 0.0001).

The structure of the porcine placenta precludes maternal antibody transfer to the fetus. Therefore, newborn piglets must ingest and absorb large amounts of colostrum during the first 24 h of life, before the gut epithelium closes (3). Given that blood IgG levels have been positively correlated with piglet survival (5,12), the objective of this study was to assess whether birth order has an effect on piglet plasma IgG content and whether eliminating this effect by preventing suckling for the first 4 h after the first piglet’s birth would increase the plasma IgG content of the late-born piglets.

Previous studies had suggested that birth order may affect early sucking, with first-born piglets having increased blood levels of IgG compared with last-born piglets (4,16), whereas others had shown only marginal (5) or no significant effect of birth order (18), and the reason is unknown. Plasma protein analysis in our study did not show differences between first-born and last-born piglets at either 24 h or 12 d postpartum. While it could be argued that the total protein values may have masked changes in IgG concentration because of a lower sensitivity of the refractometer compared with direct measurement of the IgG concentration in blood (13), others have also reported no birth-order differences in piglet IgG content as determined with a pig enzyme-linked immunosorbent assay (ELISA) kit (18). A birth-order effect on piglet IgG levels has been associated with changes in sow colostrum quality, the piglets born first likely ingesting more immunoglobulin because of a rapid decrease in colostrum IgG concentration during the first 6 h after farrowing (7,10,16).

However, others have shown that the protein content of colostrum did not decrease within 4 h of birth (13). Although speculative, it is plausible that variability in colostrum IgG content among sows and herds (17,19) underlies the differences in IgG levels between first-born and last-born piglets observed in various studies.
Restricting piglets from nursing immediately after birth has been reported to prevent a decline in IgG levels in sow colostrum (8), which has led to recommendations to remove the entire litter from the sow at parturition to normalize the colostrum intake between the first-born and last-born piglets (7,8,10). However, restricting suckling until 4 to 7 h after birth caused an overall decline in the serum Ig concentration of piglets at 12 h postpartum compared with the levels in piglets that were permitted to suckle from birth (10,12), which was attributed to either the shorter nursing period or greater weakness of the animals that were not allowed to suckle immediately (12). Our data partially agree with the results of the previous work, in that we also observed a decrease in percent plasma protein in the piglets that were separated from the dam at birth. However, removal of the piglets from the dam at birth affected neither piglet weight gain nor survival to day 12, despite the fact that the lower plasma protein levels were maintained over time. It has been argued that each piglet needs to consume at least 200 g of colostrum, with a target of at least 250 g for full passive protection (20). Likely the consumption of greater amounts will not improve piglet weight gain. The fact that piglet weight gain was not affected by birth order or treatment group in the present study suggests that the minimum threshold consumption was attained, even though the serum protein levels were reduced by restricted suckling. It would be interesting to repeat this study with sows that have a hyperprolific genotype; if colostrum is limited, an impact of restricted suckling could become apparent with the larger litters. However, with hyperprolific sows, it would not be appropriate to treat the entire litter this way; rather, the heavier or first-born piglets could be separated from the sows.

In conclusion, acquisition of passive immunity by the neonatal pigs herein was associated with the farrowing-to-suckling interval rather than with birth order and, therefore, militates against the use of a crèche to reduce preweaning mortality. This conclusion is based on the lower percent protein in the plasma of the piglets that had a delayed onset of suckling. Further studies are needed to explore directly the effects of delayed suckling on Ig levels and long-term survival.

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