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A 5-year study of the incidence and economic impact of variant infectious bursal disease viruses on broiler production in Saskatchewan, Canada

Tara Zachar, Shelly Popowich, Bob Goodhope, Tennille Knezacek, Davor Ojkic, Philip Willson, Khawaja Ashfaque Ahmed, Susantha Gomis

Abstract

While the prevalence of infectious bursal disease virus (IBDV) on chicken farms in some provinces of Canada has been documented, the economic impact of variant IBDV infection on the broiler chicken industry in Saskatchewan has not. The objectives of this study were to identify the variant strains of IBDV circulating on Saskatchewan chicken farms and evaluate their economic impact on broiler production. Infection due to IBDV was detected in 43% of Saskatchewan chicken farms, with variant strains detected in infected birds closely related predominantly to NC171, 586, and Delaware-E. Infected flocks showed an IBDV antibody titer of 4236 geometric mean (GM), whereas an antibody titer of 157 GM was measured in uninfected flocks. Infected flocks had very low (0.06) bursa-to-body-weight (BBW) ratio (an indicator of immunity) compared to high BBW ratio (0.17) in uninfected flocks, which suggests a significant immunosuppression in the former. Flocks positive for IBDV had mean mortality of 8.6% and mean condemnation of 1.5%. In contrast, mean mortality in uninfected flocks was 6.1% and mean condemnation was 1.1%. The live market weight per grow area at 37 d of age was 29.3 kg/m² in infected flocks and 34.0 kg/m² in flocks without IBDV infection. Flock mortality and condemnation rate were positively correlated with IBDV infection, whereas low BBW ratio was inversely correlated, as expected. Overall, IBDV-infected flocks had higher mortality, bursal atrophy, poorer feed conversion ratio (FCR), and decreased meat production. Our data suggest that the broiler chicken industry in Saskatchewan loses 3.9 million kilograms of meat production per year due to variant IBDV strains.

Résumé

Bien que la prévalence du virus de la maladie infectieuse de la bourse (VMIB) sur les fermes de poulets dans quelques provinces canadiennes ait été documentée, l’impact économique d’infections par des variants du VMIB sur l’industrie du poulet à griller en Saskatchewan ne l’est pas. Les objectifs de la présente étude étaient d’identifier les souches variantes du VMIB circulant au sein des fermes de poulet de la Saskatchewan et d’évaluer leur impact économique sur la production de poulets à griller. L’infection due au VMIB a été détectée sur 43 % des fermes de poulet de la Saskatchewan, avec des souches variantes détectées chez des oiseaux infectés faiblement apparentées en prédominance aux souches NC171, 586, et Delaware-E. Les troupeaux infectés présentaient une moyenne géométrique (MG) des titres d’anticorps contre le VMIB de 4236, alors que la MG des titres d’anticorps des oiseaux provenant de troupeaux non-infectés était de 157. Les troupeaux infectés avaient un très faible ratio (0.06) du poids bourse-poids corporel (BPC) (un indicateur de l’immunité) comparativement au ratio BPC élevé (0.17) dans les troupeaux non-infectés, ce qui suggère une immunosuppression significative dans les troupeaux infectés. Les troupeaux positifs au VMIB avaient un taux de mortalité moyen de 8.6 % et un taux de condamnation moyen de 1.5 %. À l’opposé, dans les troupeaux non-infectés le taux de mortalité moyen était de 6.1 % et le taux de condamnation moyen de 1.1 %. Le poids vif de marché à 37 j d’âge par surface de croissance était de 29.3 kg/m² dans les troupeaux infectés et de 34.0 kg/m² dans les troupeaux sans infection par VMIB. La mortalité dans le troupeau et le taux de condamnation étaient corrélés positivement avec l’infection par VMIB, alors qu’un faible ratio BPC était corrélé, tel qu’attendu, de manière inverse. De manière générale, les troupeaux infectés par le VMIB présentaient une mortalité plus élevée, une atrophie de la bourse, un mauvais ratio de conversion alimentaire, et une production de viande réduite. Nos données suggèrent que l’industrie du poulet à griller en Saskatchewan perd annuellement 3.9 millions de kilogrammes de production de viande à cause des souches variantes du VMIB.

(Traduit par Docteur Serge Messier)
Introduction

Infectious bursal disease (IBD) is a highly contagious immunosuppressive disease that causes serious problems for the poultry industry worldwide (1). The disease is also known as Gumboro disease as it was first recognized in the United States near the town of Gumboro, Delaware (2). Infectious bursal disease is caused by the IBD virus (IBDV), a double-stranded ribonucleic acid (RNA) virus that belongs to the genus Avibirnavirus of the family Birnaviridae (3). The IBD virus exists in classical and variant forms that are antigenically distinct (2). Emergence of variant IBDV strains has caused substantial losses in the poultry industry. Most IBDV strains circulating in the United States (2) and Canada (4) are variant strains. Infection by IBDV can cause direct economic loss due to specific mortality (1,2). The indirect economic impact of IBD is profound, however, due to IBDV-induced immunosuppression that predisposes chickens to secondary infections by bacteria, viruses, and parasites, and results in increased mortality, growth retardation, and condemnation (5-7).

Infection by the IBD virus targets the bursa of Fabricius (BF) and results in severe immunosuppression due to loss of lymphocytes in the BF (8,9). There is strong evidence that surface immunoglobulin M-bearing B-lymphocytes are the major targets for IBDV (8,10). Variant IBDV strains (11,12) in neonatal chickens can escape from the maternal antibodies (MAB) against IBDV that are produced by broiler breeders immunized with standard or classic IBDV strain vaccines (7) and induce severe bursal damage (13-16). This results in profound immunosuppression and subclinical infections (16,17). Immunosuppression is greatest when infection occurs early post-hatch and is permanent because the damaged BF does not regenerate normal immune function. As the immunosuppression resulting from an IBDV infection is often the underlying cause of respiratory and enteric disease in chickens, as well as vaccination failures, IBDV is an economically significant disease throughout the world (11). It may also precipitate a variety of other diseases or conditions that could play a role in airsacculitis, septicemia, toxemia, increasing condemnations, and decreasing broiler growth rate (10,13).

Although variant IBDV infection has recently been confirmed on Canadian chicken farms (18), its economic impact on the Saskatchewan broiler chicken industry has not been studied. The objectives of this study were to identify the incidence of variant IBDV infection and to evaluate the associated economic losses in the broiler chicken industry in Saskatchewan, Canada.

Materials and methods

Study design

This study was conducted over a 5-year period, with sample collection occurring in 2007, 2009, and 2011. According to the Chicken Farmers of Saskatchewan, there were 63 broiler farms in the Saskatchewan broiler chicken industry from 2007 to 2011. In 2007, the incidence of IBDV was studied by histologic examination of the bursa of Fabricius (BF) and polymerase chain reaction (PCR). A follow-up survey was conducted in 2009 to obtain major economic parameters, such as farm and flock (birds kept in a barn on a poultry farm) information, feed conversion ratio (FCR), and stocking density, in addition to histologic examination of BF, bursa-to-body-weight (BBW) ratio, and serology against IBDV. Another follow-up study was conducted in 2011 at those farms that were found to have been infected with IBDV in 2007 and 2009.

Incidence of variant IBDV in broiler farms in 2007

In 2007, 177 flocks managed by 58 of 63 broiler farms (92%) were chosen for this study. Bursae were collected from 40 randomly selected birds from farms with multiple barns (flocks) and from 20 birds from farms with only 1 barn (flock) at the time of processing for histologic examination throughout the 1-year period. Tissue sections of bursae were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E). A histologic score from 0 to 3 (0 = no visible lesions; 1 = mild, focal lymphoid depletion; 2 = moderate, multifocal lymphoid depletion; and 3 = moderate to severe multifocal lymphoid depletion) was assigned on the basis of the tissue reactions of the BF (Figure 1). A bursal score was assigned to each bird and a cumulative score was determined per flock with the lowest score being 0 (no bursal atrophy) and the highest score being 60 (20 birds per flock with severe bursal atrophy). All farms with bursal atrophy and 10 farms with no bursal atrophy on histologic examination were further examined by collecting bursae from 10 randomly selected birds at 19 d of age for identifying IBDV by real-time polymerase chain reaction (RT-PCR) and nucleotide sequence analysis of the viral protein 2 (VP2) hypervariable region (19). This was carried out at the Animal Health Laboratory (AHL), University of Guelph, Ontario, as described previously (4). Chickens were vaccinated for IBDV 10 to 14 d post-hatch in 4 out of 58 farms (2 farms with IBDV infection and another 2 farms without IBDV infection) with Clonevac D-78 (Intervet Canada, Whitby, Ontario).

Economic impact of variant IBDV in broiler farms in 2009

In 2009, a follow-up survey was conducted to collect data from the same 58 farms (177 barns) on the following broiler production variables: number of barns (flocks) per farm; type of barn; age and flooring of barns; total number of birds placed; stocking density; downtime between flocks; vaccines administered; FCR; age at slaughter; market weight; total birds processed; and total flock mortality, including culled birds and total condemnations at the time of processing. For each barn, data were collected from 1 production cycle from April to June, 2009. In addition to collecting production data at all participating locations, additional studies were done on some farms. At the time of processing, sets of 20 birds were randomly selected from 52 of 177 barns for IBDV serology, BBW ratio, and histologic examination of BF.

Persistence of variant IBDV in broiler farms infected since 2007

In 2011, a follow-up serological study for IBDV was conducted in 82 of 177 barns (located on 25 of 58 farms) that were confirmed to have had IBDV infection in 2007 and 2009. Confirmation of IBDV in farms was defined as the presence of 2 out of 3 criteria associated with IBDV infection, namely IBDV titer, BBW ratio, or bursal atrophy score. Infectious bursal disease (IBD) virus titers were assayed.
using commercial enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer’s instructions (IDEXX, Westbrook, Maine, USA) at the AHL, University of Guelph, Ontario.

**Statistical analysis**

Descriptive statistics and correlations among observed variables were calculated using Prism 5.0 (GraphPad Software, San Diego, California, USA). Geometric means (GM) were used to describe the central tendency of variables that were not normally distributed (antibody titer) and arithmetic means were used for the remaining variables. The indicators of infection and disease (cumulative histopathology score of the BF, BBW ratio, and IBDV GM titer) were considered individually in order to determine the classification of farms. Wilcoxon Rank-Sum Test was used to demonstrate the significance of differences in the central values of samples from each category of farm. Stepwise logistic regression was used to analyze the production variables [downtime between flocks, FCR, and meat production per square meter at 37 d of age (kg/m²)] in flocks with or without IBDV infection using Statistix 7 (Analytical Software, Tallahassee, Florida, USA).

**Results**

**Incidence of variant IBDV in broiler farms in 2007**

The histologic study conducted in 2007 classified 33 of 58 farms (57%) with minimal to no bursal damage and a mean cumulative score of 0.24 per farm. In contrast, 25 of 58 premises (43%) had
moderate to severe bursal damage, with a mean cumulative score of 40.3 per farm (Figure 1). No variant IBDVs were isolated in 10 of 33 farms with minimal to no bursal atrophy on histologic examination. In contrast, variant IBDV strains similar to NC171, 586, and Delaware E were isolated from 19-day-old broilers in 12 of the 25 farms with moderate to severe bursal atrophy (Figure 2).

Economic impact of variant IBDV in broiler farms in 2009

Production variables were collected from 58 broiler farms (177 barns) in Saskatchewan. Of 58 farms, 10 farms had a single barn, 15 farms had 2 barns, 18 farms had 3 barns, 10 farms had 4 barns, and the remaining 5 farms had 5 to 13 barns. The barns on 36 of 58 farms were 10 years old and 1 to 9 years old on 22 of 58 farms. Forty-seven of 58 farms had single-story barns, 5 of 58 farms had double-story barns, and the remaining 6 farms had both single- and double-story barns. Twenty-seven of 58 farms had concrete floors and the remaining farms had a combination of partial concrete, clay, sand, wood, or soil floors. The mean floor area of a barn was 4143 m² and ranged from 743 to 14 864 m². The median distance between processing plant and farm was 160 km, with a range of 2 to 411 km (Table I).

The mean placement on a farm was 69 048 birds, ranging from 13 000 to 217 700 birds. The mean bird density was 0.06 m²/bird, ranging from 0.04 to 0.09 m²/bird. The mean age at marketing was 37 d and ranged from 33 to 45 d. The mean market weight of a bird was 2.02 kg and ranged from 1.60 to 2.58 kg. The mean FCR was 1.83 and ranged from 1.59 to 2.22. The mean live bird production was 32.36 kg/m² and ranged from 21.48 to 49.60 kg/m². The mean mortality, including culled birds, was 7.04% and ranged from 1.26 to 20.7%. The mean BBW on a farm was 0.11% and ranged from 0.04% to 0.21%. The mean condemnation was 1.25% and ranged from 0.07% to 4.94%. Thirty-six of 58 farms maintained 9 d or longer downtime between flocks after cleaning and disinfection were completed. All broiler placements in Saskatchewan were supplied by 2 hatcheries and all broiler flocks were processed by 2 processing plants (Table I).

Infectious bursal disease (IBD) virus titers were positively correlated with the cumulative histologic score of the BF (r = 0.71, P = 0.0001), flock mortality (r = 0.47, P = 0.02), condemnation rates (r = 0.44, P = 0.02) and negatively correlated with BBW ratio (r = -0.79, P = 0.000001). The condemnation rates at the time of processing were negatively correlated with the downtime between flocks (r = -0.31, P = 0.02) and BBW ratio (r = -0.53, P = 0.005). There is no significant correlation between broiler breeder IBDV vaccination against bursal atrophy, BBW ratio, or IBDV titer (r = 0.25, P = 0.25; r = -0.27, P = 0.19; and r = 0.30, P = 0.19, respectively).

Confirmation of IBDV in farms was defined as the presence of 2 of 3 criteria associated with IBDV infection, namely IBDV titer, BBW ratio, or bursal atrophy on histologic examination, as already described. Birds from farms with IBDV infection had a GM titer of 4236 (1578 to 7466) to IBDV. In contrast, farms with no IBDV infection had a GM titer of 157 (2 to 788) to IBDV. Farms with IBDV infection had a BBW ratio of 0.06 (0.04 to 0.08), while farms with no IBDV infection had a BBW ratio of 0.17 (0.13 to 0.21). Farms with
IBDV infection had a cumulative histologic score of 49.58 (20.30 to 58.75), while farms with no IBDV infection had a cumulative histologic score of 2.11 (0.50 to 4.75). The FCR on farms with IBDV infection was 1.87, while the FCR was 1.81 in farms with no IBDV infection. The mean mortality was 8.6% (4.35% to 20.7%) in farms with IBDV infection and 6.1% (1.26% to 18.6%) on farms with no IBDV infection. The mean condemnation rate was 1.48% (0.25% to 4.94%) on farms with IBDV infection and 1.12% (0.07% to 4.6%) on farms with no IBDV infection. The live market weight per grow area (meat production) was 29.3 kg/m² on farms with IBDV infection and 34.0 kg/m² on farms with no IBDV infection (Table II).

On average, broiler producers repeat 6.5 production cycles per year. Based on our data (number of barns infected with IBDV, number of birds in barns infected with IBDV, difference in kg/m² meat production in barns with and without IBDV infection, and number of production cycles per year), the broiler chicken industry in Saskatchewan loses approximately 3.9 million kg per year due to “variant” IBDV infection. In 2014, this amount of chicken had a wholesale market value of over $13 million. (The wholesale price of chicken was $3.58/kg in 2014, according to the 2014 Annual Report of the Chicken Farmers of Canada.)

### Discussion

The economic impact of infectious bursal disease (IBD) on the chicken industry is difficult to assess due to the complex nature of losses associated with this disease. In addition to direct losses, IBDV infection-induced immunodeficiency in chickens opens the door to other viral, bacterial, and parasitic infections, thus inflicting heavy indirect losses due to increased morbidity, mortality, and condemnation (6). Moreover, IBD viruses are resistant to many disinfectants and environmental factors. Once a poultry house becomes contaminated with IBDV, viruses persist on the premises and disease tends to reappear in subsequent flocks (20). An effective IBDV prevention and control program must involve an effective surveillance strategy. This 5-year study was started in 2007 to investigate the prevalence and persistence of IBD on Saskatchewan chicken farms and assess the financial impact on the chicken industry.

In 2007, histologic study identified 43% of farms with bursal atrophy, where RT-PCR and sequencing associated these 43% of farms with variant IBDV infection. The remaining 57% of premises showed no bursal atrophy and IBDV infection was not detected. Bursal atrophy, low BBW ratio, and high IBDV titers were noted in farms with 5 or more barns, hence bursal atrophy was noted in 93 of 177 barns or broiler flocks (53%) in 2007 (Table II).

In 2009, a positive correlation of IBDV antibody titer with bursal atrophy and a negative correlation of IBDV antibody titer with BBW titer was noted. These 2 farms had a GM IBDV titer of 34.5 (26 to 43). Twenty-three of 25 farms (92%) maintained IBDV infection from 2007 to 2011.

### Persistence of variant IBDV in broiler farms infected since 2007

Of 58 farms studied, 10 out of 33 farms were without any sign of IBDV infection, i.e., no bursal atrophy, no IBDV detection, and low titers against IBDV in 2007 and 2009, and had an average IBDV GM titer of 42.6 (27 to 33e) in 2011. In contrast, 23 out of 25 farms (92%) with a history of IBDV infection in 2007 and 2009 had an average IBDV GM titer of 4063 (1104 to 9412). Only 2 out of 25 farms (8%) with IBDV infection in both 2007 and 2009 had a GM IBDV titer in 2011 that was classified as low (< 50). These 2 farms had a GM IBDV titer of 34.5 (26 to 43). Twenty-three of 25 farms (92%) maintained IBDV infection from 2007 to 2011.

### Table I. Characteristics of the Saskatchewan broiler chicken industry (2007 to 2011)

<table>
<thead>
<tr>
<th>Description of the study</th>
<th>Total</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of premises (farms) in Saskatchewan</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of premises (farms) studied</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number of barns (flocks)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number of premises with barns over 10 y of age</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Description of premises (farms)</td>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Distance between farm and processor (km)</td>
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<td>2 to 411</td>
<td></td>
</tr>
<tr>
<td>Number of barns per premise</td>
<td>3</td>
<td>1 to 13</td>
<td></td>
</tr>
<tr>
<td>Barn size m² (ft²)</td>
<td>4143 (44 600)</td>
<td>743 to 14 864 (8000 to 160 000)</td>
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</tr>
<tr>
<td>Birds per m² (ft² per bird)</td>
<td>17.3 (0.64)</td>
<td>11.5 to 26.7 (0.40 to 0.94)</td>
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</tr>
<tr>
<td>Meat production (kg/m²)</td>
<td>32.36</td>
<td>21.48 to 49.60</td>
<td></td>
</tr>
<tr>
<td>Number of birds placed on each farm</td>
<td>69 048</td>
<td>13 000 to 217 770</td>
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<tr>
<td>Mortality including culls (%)</td>
<td>7.04</td>
<td>1.26 to 20.70</td>
<td></td>
</tr>
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<td>Feed conversion ratio (FCR) (feed offered/weight gain)</td>
<td>1.83</td>
<td>1.59 to 2.22</td>
<td></td>
</tr>
<tr>
<td>Number of birds processed</td>
<td>64 098</td>
<td>10 860 to 207 830</td>
<td></td>
</tr>
<tr>
<td>Processed weight per farm (kg)</td>
<td>135 538</td>
<td>21 806 to 440 653</td>
<td></td>
</tr>
<tr>
<td>Live weight at processing (kg)</td>
<td>2.02</td>
<td>1.60 to 2.58</td>
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</tr>
<tr>
<td>Condemnation rate (%)</td>
<td>1.25</td>
<td>0.07 to 4.94</td>
<td></td>
</tr>
<tr>
<td>Cumulative histopathology score of bursa of Fabricius (BF) per farm</td>
<td>29.81</td>
<td>0.50 to 58.75</td>
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</tr>
<tr>
<td>Bursa-to-body-weight (BBW) ratio</td>
<td>0.11</td>
<td>0.04 to 0.21</td>
<td></td>
</tr>
<tr>
<td>IBDV titer [geometric mean (GM)]</td>
<td>2137</td>
<td>2 to 7466</td>
<td></td>
</tr>
<tr>
<td>Downtime between flocks (d)</td>
<td>12</td>
<td>1.5 to 22</td>
<td></td>
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</table>
Table II. Production variables and indicators of infection on farms with or without variant infectious bursal disease viruses (vIBDVs)

<table>
<thead>
<tr>
<th>Description of flocks</th>
<th>Without vIBDV</th>
<th>With vIBDV</th>
<th>Percentagea</th>
<th>Without vIBDV</th>
<th>With vIBDV</th>
<th>Percentagea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of farms</td>
<td>33</td>
<td>25</td>
<td>(57%)</td>
<td>84</td>
<td>93</td>
<td>(53%)</td>
</tr>
<tr>
<td>Number of barns</td>
<td>84</td>
<td>93</td>
<td>(47%)</td>
<td>19</td>
<td>17</td>
<td>(47%)</td>
</tr>
<tr>
<td>Number of farms with barns &gt; 10 y of age</td>
<td>19</td>
<td>17</td>
<td>(53%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indicators of infection and diseasec</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative histopathology score of the BFb</td>
<td>2.11</td>
<td>49.58</td>
<td>0.5 to 4.75</td>
<td>20.3 to 58.75</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Bursa-to-body-weight (BBW) ratio (percentage)</td>
<td>0.17</td>
<td>0.06</td>
<td>0.13 to 0.21</td>
<td>0.04 to 0.08</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>IBDV (GM titer)</td>
<td>157</td>
<td>4 236</td>
<td>2 to 788</td>
<td>1578 to 7466</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Production variablesd</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Downtime between flocks (days)</td>
<td>12</td>
<td>11</td>
<td>2 to 22</td>
<td>4 to 20</td>
<td>NS</td>
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<tr>
<td>Feed conversion ratio (FCR)</td>
<td>1.81</td>
<td>1.87</td>
<td>1.59 to 2.22</td>
<td>1.74 to 2.04</td>
<td>NS</td>
<td></td>
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<tr>
<td>Meat production (kg(\text{kg}/\text{m}^2))</td>
<td>34.0</td>
<td>29.3</td>
<td>18.1 to 54.0</td>
<td>21.9 to 39.0</td>
<td>0.83 (CI .72 to 0.95)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

* a The number in parentheses indicates the portion of the grand total in each disease category.
* b Cumulative histopathology score from 2007 or mean of scores from 2007 and 2009 if farms were sampled in both years.
* c Since each of the indicators of infection and disease is considered individually in order to determine the classification of farms, Wilcoxon Rank-Sum Test is used to demonstrate the significance of differences in the central values of samples from each category of farm.
* d Stepwise logistic regression was used to analyze the production variables. The odds of a “vIBDV present” disease occurrence are 0.83 (83% of) less for a flock with a meat production increase of 1 kg/m\(^2\). The difference in the means (34.0 to 29.3 = 4.7 kg/m\(^2\)) indicates the amount of loss due to the disease.

BF — Bursa of Fabricius; GM — Geometric mean; NS — Not significant; CI — Confidence interval.

ratio were observed. Bursa-to-body-weight (BBW) ratio was also negatively correlated with condemnation rates. Phylogenetic analysis of VP2 sequences (19) obtained from the viruses isolated from the BF of 19-day-old broilers revealed that the IBDV strains circulating on Saskatchewan chicken farms with bursal atrophy are variant strains closely related to NC171, 586, and Delaware-E (Figure 2).

Flocks at premises infected with variant IBDV in 2007 and 2009 were tested again for the presence of IBDV antibodies in 2011 and 92% of these premises were serologically positive. It appeared that IBDV infection persisted for at least 4 y in broiler farms once they were infected. Infectious bursal disease (IBD) virus infection was controlled in only 2 of 25 farms that had few (3 or 4) barns. This indicates that control of IBDV infection was manageable in small farms with few barns. Furthermore, the incidence of IBDV infection remained very low (10%; 1 of 10 farms) in farms with a single broiler barn. In contrast, farms with 4 or more barns had a much higher incidence (87%; 13 of 15 farms) of IBDV infection.

Farms with IBDV infection had higher IBDV titers, poorer FCR, higher mortality, more bursal atrophy, higher condemnation rates, and less meat production than premises without IBDV infection. Farms with no detectable IBDV infection had better economical parameters, including better feed conversion ratio (FCR), lower mortality, and decreased condemnation rates. The live market weight per grow area was 29.3 kg\(\text{kg}/\text{m}^2\) in farms with IBDV infection and 34.0 kg\(\text{kg}/\text{m}^2\) in farms with no IBDV infection. Broiler farms in Saskatchewan follow 6.5 production cycles per year. Since IBDV infection was noted in 53% of barns [25 of 58 (43%) of farms] in the Saskatchewan broiler industry, the projected total production loss was 3.9 million kg in barns infected with IBDV.

While the present data showing significant economic losses due to IBDV infection is in agreement with previous studies (21), it is difficult to determine whether all the losses were due to IBDV alone or there were other contributing factors related to management, environment, and feed. We have seen a reduced weight gain of 160 to 200 g per bird at 35 d old in commercial broilers after experimental infection with NCI71 compared to control birds receiving no IBDV in our level-2 animal isolation facility (22).

Less downtime between flocks was correlated with increased condemnation rates. Infectious bursal disease (IBD) virus titer was not correlated with downtime, however, which reflects the persistence of IBDV in a broiler barn for a long duration, as reported previously (23). According to the survey data, the Saskatchewan industry practiced downtime of 1.5 to 22 d between flocks. It is likely that this duration of downtime is not sufficient to reduce IBDV load in a barn, especially when thorough cleaning and disinfection are not practiced. Infectious bursal disease (IBD) virus is very resistant to most disinfectants and environmental factors and persists for months in contaminated poultry houses and for weeks in water, feed, and fecal droppings (23). Although barns were cleaned and disinfected after each cycle of birds in the study area, we have shown that IBDV infection was more prevalent in older barns and barns with no concrete floors probably because
these types of facilities are more difficult to thoroughly clean and disinfect.

Based on our data, it is concluded that unique variant IBDV strains were prevalent on Saskatchewan farms and probably attributed to substantial economic losses of about 3.9 million kg per year in the broiler chicken industry in Saskatchewan. Furthermore, it may suggest that current broiler and broiler breeder vaccination programs are not effective for controlling variant IBDV infections. Studies to identify potential vaccine candidates to control variant IBDV in Saskatchewan are urgently needed.

**Acknowledgments**

Financial grants were provided by the Chicken Farmers of Saskatchewan (Saskatchewan Chicken Industry Development Fund), Saskatchewan Agriculture Development Fund, and Natural Sciences and Engineering Research Council. Special thanks are extended to all broiler producers in Saskatchewan who participated in this study. Special thanks also to Sofina Foods Inc. (Lilydale Inc.), Wynyard, Saskatchewan and Prairie Pride Natural Foods Ltd., Saskatoon, Saskatchewan for helping with sample collection. Thanks to Drs. Azita Taghavi, Angela Oranchuk, Brenda Bryan, and Tara Funk for assisting with data collection and histologic examinations.

**References**

Detection and phylogenetic analysis of bovine papillomavirus in cutaneous warts in cattle in Tamaulipas, Mexico

Edith Rojas-Anaya, Antonio Cantú-Covarrubias, José Francisco Morales Álvarez, Elizabeth Loza-Rubio

Abstract

Papillomas occur more frequently in cattle than other domestic animals. The causal agent of bovine papillomatosis is a virus that belongs to the family Papillomaviridae. In Tamaulipas, Mexico, the virus is considered a serious problem and has impeded the export of cattle to the United States, resulting in serious economic losses. Owing to the lack of information regarding the subtypes of papillomaviruses that infect cattle in Mexico, the aim of this study was to determine the subtypes in Tamaulipas. Fifty-two warts were analyzed with the use of polymerase chain reaction (PCR) involving primers that amplify the E7 gene of bovine papillomavirus (BPV). The PCR products were sequenced to differentiate the BPV-1 and BPV-2 subtypes. The sequencing quality was determined with the use of MEGA 6.0 software. Comparison of the Tamaulipas sequences with those of known BPV types by means of the MUSCLE algorithm showed that 53% of the former were BPV-1 and 47% were BPV-2. The distribution of the 2 subtypes in the cattle was homogeneous. This study demonstrated the presence of BPV-1 and BPV-2 in cattle from Tamaulipas and constitutes the first molecular characterization of papillomas in Mexico.

Résumé

Les papillomes sont rencontrés plus fréquemment chez les bovins que chez n’importe quelle autre espèce domestiques. L’agent causal de la papillomatose bovine est un virus appartenant à la famille Papillomaviridae. Dans l’état mexicain de Tamaulipas le virus est considéré comme un problème sérieux et a empêché l’exportation de bovin vers les États-Unis d’Amérique, causant ainsi des pertes économiques importantes. Étant donné le manque d’information concernant les sous-types de papillomavirus qui infectent les bovins au Mexique, l’objectif de l’étude était de déterminer les sous-types présents dans l’état de Tamaulipas. Cinquante-deux verrues ont été analysées par réaction d’amplification en chaine par la polymérase (ACP) à l’aide d’amorces amplifiant le gène E7 du papillomavirus bovin (PVB). Les produits de l’ACP ont été séquencés afin de différencier les sous-types PVBA-1 et PVBA-2. La qualité du séquençage fut déterminée à l’aide du logiciel MEGA 6.0. La comparaison des séquences obtenues pour l’état de Tamaulipas avec celles des types connus de PVB par l’algorithme MUSCLE a permis de démontrer que 53 % étaient des PVB-1 et 47 % de PVB-2. La distribution des deux sous-types chez les bovins était homogène. La présente étude démontre la présence de PVB-1 et PVB-2 chez les bovins de Tamaulipas et constitue le premier rapport sur la caractérisation moléculaire des papillomes au Mexique.

Introduction

Bovine papillomavirus (BPV) is a nonenveloped, icosahedral virus with a double-stranded circular DNA genome of approximately 8000 base pairs (bp) containing 5 or 6 open reading frames (ORFs) that are expressed early during infection and 2 ORFs that are expressed late during infection. Because BPV causes tumor-like lesions in the skin and mucosa (1), it is the carcinogenic virus most used as a study model in research pertaining to cattle. These viruses are divided into 29 genera according to the L1 gene sequence (2) and 12 subtypes, which include the genera Deltapapillomavirus, Xipapillomavirus, and Epsilonpapillomavirus, the last being a nonclassified BPV-7 (2–4). This classification is based on the lesion (i.e., warts and/or tumors in the skin and mucosa) or a tumor association (5). Subtypes 1 and 2 have been more widely studied than the other types, and only a few studies have reported the prevalence of the other virus types. Most molecular epidemiologic studies have demonstrated coinfection by 2 or more BPV types in the same sample (6,7).

Papillomaviruses are classically described as epitheliotropic. The lesions are mainly mucocutaneous warts and are in large numbers as a result of highly productive infection. These warts are often visible in the skin of the scalp, tongue, teats, penis, oral cavity, and upper digestive tract (8). Furthermore, the virus has been detected in different bodily fluids, such as semen, urine, blood, and milk (8). The presence of the virus has also been reported in the bladder, placenta, and lymphocytes, which may play roles as viral reservoirs (9–11).

To date, there are no reports of a predilection of any viral subtype for animals of a specific gender or age. Therefore, it is possible to find different types of lesions in a single animal. Although it is...
generally thought that papillomaviruses are species-specific, BPV has recently been detected in various livestock and bison (12), water buffaloes (13), giraffes, zebras, and antelopes (14), yaks (15), horses and donkeys (16,17), tapirs (18), and other species. Papillomas can disappear or progress to malignant tumors, and their progression is associated with some specific viral subtypes as well as expression of the early (E) genes of the virus (19). Interaction of the E5, E6, and E7 oncoproteins with the host DNA is associated with the progression of a viral infection to a tumor. The subtypes BPV-1, BPV-2, and BPV-4, which express these genes, are associated with malignant tumors (20). The L1 gene target is widely used for the molecular detection of BPV (21) because it is conserved and present in all papillomaviruses. Because the E7 gene encodes an oncoprotein, this gene was used in this study as an indicator of the viral subtypes causing tumor-associated papillomas and to identify animals in which the infection might progress to a malignant neoplasm.

Bovine papillomatosis is widely distributed throughout the world. No study conducted in Mexico has indicated which papillomavirus subtypes are present in the country’s herds, even though the presence of the disease is known. Veterinarians have observed virus-associated lesions in cattle in the major agricultural regions, including Tamaulipas, which ranks third in terms of the national production of cattle, accounting for 16.38% (22). In fact, this state is one of the most important localities for cattle exports to the United States, and in recent years this virus has become an animal health problem that has caused considerable economic losses. However, there are
no epidemiologic data for bovine papillomatosis in Tamaulipas, and
the only information known is that the incidence of this disease has
increased considerably in Tamaulipas over the last 7 y. Thus, there
is a strong need to identify which BPV subtypes are circulating
within this geographic area. The purpose of this study was there-
fore to determine the subtypes of BPV that are present in cattle in
Tamaulipas and to analyze them phylogenetically.

Materials and methods
This study constituted a descriptive and cross-sectional investiga-
tion with respect to the prevalence of BPV infection in the population
or subgroups of animals sampled within the population at a given
time. Nonprobabilistic convenience sampling was done (23), and
the data were analyzed with use of the SPSS descriptive statistics
package, version 17.0 (SPSS, Chicago, Illinois, USA).

Sample collection
Biopsy samples of skin warts approximately 0.5 to 1 cm in diam-
eter were collected from different areas of the bodies of 52 female
and male cattle chronically affected with cutaneous papillomatosis in
2 regions of Tamaulipas: in the north, the municipalities of Gustavo
Díaz Ordaz and Reynosa; and in the southeast, Soto La Marina and
Aldama (Figure 1). Some samples were fixed in formalin and embed-
ded in paraffin by routine methods. Sections from 3 different types
of lesions were stained with hematoxylin and eosin.
The study protocols were approved by the Institutional Animal
Care and Use Committee of the Centro Nacional de Investigación
Disciplinaria en Microbiología Animal (approval 002-2014). All
efforts were made to minimize animal suffering and distress accord-
ing to the guidelines of Mexican Regulation NOM-033-ZOO-1995 for
the euthanasia of domestic and wild animals.

DNA extraction
A mechanical homogenizer (Qiagen, Hilden, Germany) was used
to extract DNA, which was purified with the QIAamp DNA Mini
Kit (Qiagen) extraction kit according to the manufacturer’s recom-

mended protocol with some modifications. Briefly, 100 mg of each
wart sample in 200 µL of lysis buffer ATL and 20 µL of proteinase
K was homogenized by vortexing. The mixture was incubated at
56°C for approximately 2 h. After the samples were centrifuged,
200 µL of buffer AL was added, and the samples were incubated at
70°C for 10 min. Ethanol (200 µL) was added, and the mixture was
placed in a mini QIAamp spin column and centrifuged at 6000 × g
for 1 min. The column was washed with buffer AW1 and centrifuged
as described previously. Finally, the DNA was eluted with 200 µL of
eution buffer AE. The quantity and quality of the obtained DNA
were determined spectrophotometrically. The DNA was then stored
at −20°C until used.

Detection of the E7 gene
The E7 BPV gene (383 bp) was cloned as a positive control for
polymerase chain reaction (PCR). For detection of this gene the
following primers were designed in house by amplifying a 203-bp
fragment of the gene: E7BPV+, TAG GAA GCG AGG CWC AKA
TA; and E7BPV−, CYC GMG GAC AAC ACA GG. The FastStart

Table I. Prevalence of papillomatosis in breeding cattle in
2 regions of Tamaulipas, Mexico

<table>
<thead>
<tr>
<th>Region</th>
<th>Number (and %) of animals assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northeast</td>
<td></td>
</tr>
<tr>
<td>Reynosa</td>
<td>220</td>
</tr>
<tr>
<td>Díaz Ordaz</td>
<td>40</td>
</tr>
<tr>
<td>Southeast</td>
<td></td>
</tr>
<tr>
<td>Aldama</td>
<td>764</td>
</tr>
<tr>
<td>Soto La Marina</td>
<td>649</td>
</tr>
</tbody>
</table>

PCR High-Fidelity PCR System (Roche, Mannheim, Germany) was
used with the following: 5 µL of 10× PCR buffer with magnesium,
10 mM of each primer, 2.5 µL of High-Fidelity Enzyme Blend, 200 µM
deoxynucleotide triphosphates, 2 µL of DNA, and water of
molecular-biology grade. The samples were amplified in an iCycler
(BioRad, Hercules, California, USA) with the following thermocy-
cling protocol: 95°C for 5 min; 35 cycles of 95°C for 30 s, 57°C for 30 s,
and 72°C for 30 s; and 72°C for 3 min. The amplification products
were purified on 1.5% agarose gels with use of the QIAquick Gel
Extraction Kit (Qiagen) according to the manufacturer’s instructions.
The purified products were quantified and visualized on a 1.5% agar-
ose gel to determine their quality and the purified DNA was then
sent for sequencing to the Instituto de Biotecnología, Universidad
Nacional Autónoma de México and the quality of sequences was
evaluated with MEGA 6.0 software (24).

Phylogenetic analysis
Comparison and phylogenetic analyses were done with a 203-bp
fragment of the E7 gene. The nucleotide sequences of all BPV sub-
types of different species and geographic origins obtained from
GenBank (National Center for Biotechnology Information, Bethesda,
Maryland, USA) were included in an alignment done with the algo-

msequence comparison by log expectation),
which involved calculating the distance between pairs of nucleotides
(25). From the nucleotide sequences in this dataset, a phylogenetic
tree was constructed by means of the maximum likelihood method
with the JC + G + I model. A consensus tree was obtained from
1000 bootstrap replicates.

Data analysis
The case data were analyzed by nonparametric tests, with frequen-
cies used to determine the prevalence per herd and municipality, and
a Chi-square test was conducted to determine statistical associations.

Results
The prevalence of bovine papillomatosis in the cattle of
Tamaulipas (Table I) ranged from 2% to only 5% in the northeastern
region, averaging 3% in Reynosa and 5% in Díaz Ordaz, whereas it
ranged from 2% to 70% in the southeastern region, averaging 9% in
Soto la Marina and 14% in Aldama. The virus had no predilection for

The prevalence of bovine papillomatosis in the cattle of
Tamaulipas (Table I) ranged from 2% to only 5% in the northeastern
region, averaging 3% in Reynosa and 5% in Díaz Ordaz, whereas it
ranged from 2% to 70% in the southeastern region, averaging 9% in
Soto la Marina and 14% in Aldama. The virus had no predilection for
Table II. Features of the 19 cattle with wart samples positive for subtypes of bovine papillomavirus (BPV)

<table>
<thead>
<tr>
<th>Locality</th>
<th>Gender</th>
<th>Breed</th>
<th>Herd</th>
<th>Age (y)</th>
<th>Lesion morphology</th>
<th>Lesion location</th>
<th>BPV type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reynosa</td>
<td>Female</td>
<td>Charolais</td>
<td>Beef</td>
<td>48</td>
<td>Cauliflower-like</td>
<td>Flank</td>
<td>2</td>
</tr>
<tr>
<td>Diaz Ordaz</td>
<td>Male</td>
<td>Charolais</td>
<td>Semental</td>
<td>16</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>1</td>
</tr>
<tr>
<td>Aldama</td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>28</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>29</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Charolais/cebu</td>
<td>Beef</td>
<td>14</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Simbrah</td>
<td>Beef</td>
<td>16</td>
<td>Cauliflower-like</td>
<td>Head, neck, flank</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>14</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>13</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>11</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>2</td>
</tr>
<tr>
<td>Soto La Marina</td>
<td>Female</td>
<td>Charolais/cebu</td>
<td>Beef</td>
<td>14</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot, abdomen</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Charolais/cebu</td>
<td>Beef</td>
<td>16</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Charolais/cebu</td>
<td>Beef</td>
<td>15</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simmental</td>
<td>Beef</td>
<td>11</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot, foot</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>40</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Simbrah</td>
<td>Beef</td>
<td>10</td>
<td>Cauliflower-like</td>
<td>Head, neck, flank</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>11</td>
<td>Cauliflower-like</td>
<td>Head, neck</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Simbrah</td>
<td>Beef</td>
<td>10</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot, flank</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Simbrah</td>
<td>Beef</td>
<td>11</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot, abdomen</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Simbrah</td>
<td>Beef</td>
<td>12</td>
<td>Cauliflower-like</td>
<td>Head, neck, foot, flank</td>
<td>1</td>
</tr>
</tbody>
</table>

The 19 and BPV-2 in 9 (47%). Hyperplasia was present primarily in the animals infected with the BPV-2 subtype. The dendrogram obtained (Figure 3) indicated 2 different clades, corresponding to subtypes 1 and 2. It is noteworthy that the Mexican sequences classified as subtype 1, which originated from the samples collected in Soto La Marina and Aldama, were grouped in a clade different from that containing the sequences classified as subtype 1 that were published in GenBank, which originated in Japan, the United States, Iraq, and Austria. No differences were found in the geographic distribution of the sequences grouped as subtype 2, which fell into 3 different sub-clades; these sequences corresponded to those of the samples obtained from Aldama, Soto La Marina, and Reynosa. The presence of both viral subtypes on the same farm was also demonstrated. The distance matrix showed little difference among the Mexican sequences but significant differences between the sequences of subtypes 1 and 2.

Discussion

Before this study, there was no published information regarding the subtypes of BPV in any state of Mexico, despite the economic importance of bovine papillomatosis. We used PCR to assess the presence of BPV-1 and BPV-2 in samples of hyperplastic and macroscopic warts collected from chronically infected adult cattle in different herds in Tamaulipas, Mexico.

Of the 52 cattle evaluated by wart sampling, 19 (36%) were positive for 1 of 2 subtypes of BPV. Campo et al (26) detected BPV-2 DNA in natural tumors of immunosuppressed animals and demonstrated an association between BPV-2 and bovine bladder tumors. Borzacchiello et al (27) also demonstrated an association between...
BPV-2 and bovine bladder tumors and detected BPV-2 in samples with and without neoplastic change. Histopathological analysis of lesions is important for identifying intraepithelial tumors associated with oncogenic viruses such as BPV (28), and this analysis shows whether the virus has a predilection for anatomic areas related to a specific viral type. The histopathological findings reported in the literature for BPV include acanthosis, hyperkeratosis, parakeratosis, papillomatosis, and koilocytosis (29). We observed similar features in this study (Figure 2) and found that hyperplasia was present primarily in the animals infected with the BPV-2 subtype.

Both BPV-1 and BPV-2 have been reported to be associated with skin warts and fibropapillomas in cattle and sarcoids in horses. Owing to the presence of oncoproteins, BPV is genetically unstable. The E5, E6, and E7 genes, which are related to the transformation of cells, are found in BPV-1. The function of each viral oncogene depends on the viral subtype. The E5 gene is highly conserved among the group of BPVs, is the main transforming gene (26), and induces cell transformation, proliferation, and survival (30). Not all oncogenes are present in all BPV subtypes, some subtypes being deficient in E6, regardless of how the infection occurs (31). In contrast, E7 is a zinc-binding protein, and E5 and E7 coexpression increases the transforming capacity of BPV (32). The oncogene E7 leads to cellular transformation, degradation of the pRb tumor suppressor, deregulation of the cell cycle, and tumorigenesis (29,33–35). We were interested to determine the presence of the E7 gene in the sampled warts because of this gene’s association with cellular

Figure 3. Maximum likelihood tree for bovine papillomavirus (BPV). The strains in blue are BPV-1, and the strains in red are BPV-2.
transformation. The E7 gene is important in oncogenesis, and independent of deletions or mutations its expression is related in some degree to tumor formation. Therefore, we selected this gene as the target in our molecular characterization study. It is more conserved and likely cannot be used to discriminate between some subtypes, whereas the E6 gene is not present in all subtypes.

The phylogenetic tree showed that the sequences of the detected BPV subtypes 1 and 2 were closest to those of North American strain type 1. Thus, these strains likely circulate between Mexico and the United States because of trade relations and may even infect wildlife species that can move freely across the border and have not been studied. The presence of both subtypes in different regions of Tamaulipas could indicate that the animals had been moved indiscriminately across the state. With respect to BPV-2, the tree showed that isolates from China and Mexico have a common ancestor (accession no. M02219), but its origin is not specified in GenBank. This finding was corroborated by the distance matrix, which revealed a value less than 0.02 for the aforementioned isolate.

The main finding of this study is identification of the BPV subtypes in beef cattle with papillomatosis in Tamaulipas, Mexico. This identification, the first molecular characterization of BPV-1 and 2 in papillomas done in Mexico, is of epidemiologic importance because of the economic value related to the export of cattle from Tamaulipas to the United States: in 2014 alone, 138,094 cattle were exported (33). Biosecurity measures should be implemented in herd management to prevent or reduce the overall transmission of these subtypes among the animals.

Acknowledgments

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References


Safety and efficacy of a novel European vaccine for porcine reproductive and respiratory virus in bred gilts

Michael D. Piontkowski, Jeremy Kroll, Francois-Xavier Orveillon, Christian Kraft, Teresa Coll

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) can be devastating to commercial breeding operations. The objective of this study was to evaluate a novel European PRRSV vaccinal strain for safety and efficacy in bred gilts. In 2 experiments, 110 gilts were vaccinated intramuscularly and the vaccine was evaluated for safety and efficacy. Gilts in Experiment 1 were vaccinated for local and systemic reactions and gilts in both experiments were observed for clinical signs of disease through farrow. In both experiments, piglet clinical observations, piglet average daily weight gain (ADWG), gilt serology [determined by enzyme-linked immunosorbsent assay (ELISA)], gilt and piglet viremia [determined by quantitative real-time polymerase chain reaction (qPCR)], as well as piglet lung lesion scores and PRRS virus in lung tissue (qPCR) were determined. The vaccine was shown to be safe to be as there were no significant differences among groups in either experiment. Efficacy was established in Experiment 2 as both vaccinated groups were associated with desirable significant differences in percentage of gilts with abnormal clinical findings; gilt viral load post-challenge [day 125, day of farrowing (DOF), and DOF + 13]; percentages of alive, healthy live, weak live, and mummified piglets per litter at farrowing and weaning; percentage of piglets per gilt that were positive for viremia; percentage of piglets per gilt with clinical disease; and piglet viral load on DOF. It was concluded that a vaccine formulated from the PRRSV modified live virus (MLV) strain 94881 is a safe and effective method of protection against the detrimental effects of virulent PRRSV infection in breeding female pigs.

Résumé

Le virus du syndrome reproducteur et respiratoire porcin (VSRRP) peut être dévastateur pour les opérations de reproduction commerciales. L’objectif de la présente étude était d’évaluer l’innocuité et l’efficacité d’une nouvelle souche vaccinale européenne du VSRRP chez des cochettes saillies. Lors de deux expériences, 110 cochettes ont été vaccinées par voie intramusculaire afin d’évaluer l’innocuité et l’efficacité du vaccin. Les cochettes de l’Expérience 1 ont été évaluées pour la présence de réactions locales et systémiques et les cochettes dans les deux expériences ont été observées pour vérifier la présence de signes cliniques de maladie jusqu’au moment de la mise-bas. Lors des deux expériences on nota les observations cliniques des porcelets, le gain de poids quotidien moyen des porcelets (GMQ), les titres sérologiques des truies [determinés par épreuve immuno-enzymatique (ELISA)], la virémie chez les cochettes et les porcelets [determinées par réaction d’amplification en chaine par la polymérase quantitative (qACP)], ainsi que par les pointages de lésions pulmonaires des porcelets et la quantité de virus SRPP dans le tissu pulmonaire (qACP). Le vaccin s’est avéré sécuritaire et il n’y avait pas de différence significative entre les groupes dans les deux expériences. L’efficacité a été établie lors de l’Expérience 2 alors que les deux groupes vaccinés ont été associés à des différences significatives souhaitées dans le pourcentage de cochettes avec des trouvailles cliniques anormales; dans la charge virale post-challenge par cochette [jour 125, jour de la mise-bas (JMB), et JMB + 13]; pourcentages de porcelets vivants, vivants en santé, vivants faibles, et momifiés par portée au moment de la mise-bas et au sevrage; pourcentages de porcelets par truie qui étaient positifs pour une virémie; pourcentage de porcelets par cochette avec une maladie clinique; et charge virale des porcelets au JMB. Il a été conclu qu’un vaccin formulé à partir de la souche 94881 du VSRRP vivant modifié est une méthode sécuritaire et efficace de protection contre les effets néfastes d’une infection par le VSRRP chez des porcs femelles de reproduction.

(Traduit par Docteur Serge Messier)

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) can be devastating to breeding herds due to losses resulting from reproductive failure, i.e., reduced fertility, abortions, and premature farrowing (1–8), as well as delayed return to estrus (2,4). Piglets born to affected dams suffer from increased weakness at birth, decreased growth rates, increased susceptibility to respiratory infections, and higher pre-weaning mortality (1,2,6,7,9). Vaccination before breeding is considered to be of value for decreasing these detrimental effects. The 2 main types of commercially available vaccines, attenuated or modified live virus (MLV) and inactivated or killed virus (KV), confer various levels of protection. Several factors, including type of vaccine used (10), viral diversity (10,11), and timing (12,13), can...
directly affect the safety and efficacy of vaccinations. It is commonly accepted that inactivated vaccines are safe to administer to breeding animals (2,6,14) as they do not spread to other animals in the herd and do not induce reproductive failure in bred females (6) and are thus primarily recommended for immunizing animals used for reproduction.

Concerns have been raised about the safety of MLV vaccines due to the risk of vertical and horizontal transmission of vaccine virus (1,6,12,14–17) and the potential for reduced reproductive performance when administered during gestation (12). Despite safety concerns, MLV vaccines are commonly administered during gestation as they have been reported to provide more protection against infection than inactivated vaccines when faced with heterologous challenge (11,16,17). It has been reported that inactivated vaccines did not prevent viremia and/or clinical signs of PRRS (2,6,17,18). Vaccination with an MLV vaccine, however, has been shown to protect gilts from viremia and reduce congenital infection of piglets and pre- and post-natal death (2,6,16). In clinical trials, both attenuated (2,6,8,10,19) and inactivated (8,10,18) vaccines have been proven to provide effective protection against homologous challenge. Due to significant viral diversity, however, all challenge situations in the field could be considered heterologous (20).

Specifically, there are 2 main genotypes of PRRS virus, European (EU, Type 1) or North American (NA, Type 2) (3,8,15,19,21–25), which are further divided into multiple clusters (24). Since new strains continue to be found, including highly pathogenic strains (23), it is important that vaccines offer cross-protection against heterologous strains. In addition to the type of vaccine used, timing of vaccine administration is also important.

The objective of this study was to evaluate the potential for safety and efficacy of a new PRRSV modified live virus (MLV) vaccine based on a novel strain [94881, US patent #8,765,142 B2, European Collection of Cell Cultures (ECACC) accession # ECACC 11012501 (parent strain) and ECACC 11012502 (attenuated strain)] (26) when administered to either bred gilts or to breeding-age gilts before conception and the subsequent heterologous challenge.

Materials and methods

Animals and study design

Protocols were reviewed and approved by the contract research organization’s Institutional Animal Care and Use Committee before study initiation. Two experiments were conducted to establish the safety (Experiment 1) and efficacy (Experiment 2) of the test vaccine in gilts. A total of 16 bred and 94 non-bred PRRSV-negative [enzyme-linked immunosorbent assay (ELISA) sample to positive (S/P) ratio of < 0.4], commercial mixed-bred, bred gilts were used for Experiment 1 and Experiment 2, respectively. For Experiment 1, a statistician randomly assigned gilts to either group 1A (n = 8) or 1B (n = 8) before day 0 and each group was then housed in 2 separate rooms. All gilts were 90 ± 3 d of gestation at the time of first vaccination and were clinically healthy. On days 0 and 14, gilts in group 1B were administered the test vaccine at approximately 10 times (10×) overdose, while gilts in group 1A received a placebo. Gilts in groups 1A and 1B were monitored for local and systemic reactions, including serology and viremia testing, to establish vaccine safety. All gilts in Experiment 1 subsequently farrowed on days 22 to 30 and piglets were monitored for number of piglets born live, dead (stillborn), weak, mummified, and crushed/mortality, as well as for viremia, average daily weight gain (ADWG), and lung pathology.

For Experiment 2, a statistician randomly assigned 94 healthy, non-bred, approximately 8-month-old, commercial mixed-breed gilts to 1 of 4 treatment groups (group 2A, n = 28; group 2B, n = 28; group 2C, n = 28; and group 2D, n = 10). Gilts in groups 2B and 2C were housed with their respective groups in 4 rooms (2 rooms per group) at the test facility for the duration of the trial, while gilts in groups 2A and 2D were housed together in a single room at an alternate facility. On day 0, approximately 28 d before breeding, gilts in groups 2A and 2D were administered a placebo product, while gilts in groups 2B and 2C were vaccinated with a low and high titer of the test vaccine, respectively. After vaccination, gilts were observed for local and systemic reactions. Gilts were tested for PRRSV serology and viremia, synchronized for breeding, artificially inseminated (AI), and evaluated for pregnancy by ultrasound. A total of 53 healthy bred gilts, 16 gilts each from groups 2A, 2B, and 2C and 5 gilts from group 2D (negative control group) were randomly selected and subsequently enrolled into the challenge portion of the trial. Gilts in groups 2A to 2C were challenged with a heterologous [88.8% sequence homology at open reading frame (ORF) 5], Type 1 virulent strain of PRRSV and monitored for clinical signs of disease, including abortions and rectal temperatures. Gilts subsequently farrowed on days 134 to 147 and piglets were monitored for number of piglets born live, dead (stillborn), weak, mummified, and crushed/mortality, as well as for viremia and ADWG. Additionally, all piglets that were either born dead or died before DOF + 20 were necropsied and evaluated for lung pathology.

For the vaccination phase of Experiment 2, gilts in Groups 2B and 2C were housed by group in Biosafety Level 2 (BSL2) rooms at the test facility, while gilts in Groups 2A and 2D were housed together at an alternate facility until day 85 when the gilts were housed in rooms at the test facility. For Experiments 1 (entire trial) and 2 (beginning on day 85), bred gilts were housed in BSL2 rooms according to group and were placed in individual elevated crates. Crates were approximately 5 ft × 7 ft in size, were equipped with a nipple waterer, feeder, and plastic slatted flooring that was elevated above the floor, and did not allow for nose-to-nose contact. For biosecurity purposes, each BSL2 room was separately ventilated with both HEPA filters and mechanical ventilation, animal services staff were required to shower and don clean clothing before entering each room, and appropriate measures were taken to prevent accidental cross-contamination from vaccines and/or challenged gilts to negative control gilts. All gilts were fed an age-appropriate, commercially available, non-medicated gestation or lactation ration (Heart of Iowa Cooperative, Roland, Iowa, USA) as appropriate for their condition. Food and water were available ad libitum.

Serology and viremia assays

Blood was collected by jugular venipuncture from gilts in Experiments 1 and 2 on days 0, 14, DOF, and DOF + 21 and on
days 0, 7, 14, 21, 56, 84, 118, 125, 132, DOF, DOF + 7, DOF + 13, and DOF + 20, respectively. Venous whole blood was collected from piglets in Experiment 1 on the day of birth and DOF + 21 and from piglets in Experiment 2 on the day of birth, DOF + 7, DOF + 13, and DOF + 20, or when any piglet was found dead. Additionally, blood was collected from each mumified or stillborn piglet, but if this was not possible, thoracic or abdominal fluid was collected. Blood samples were processed for serum and fluids were aliquoted into appropriate tubes and held at either 2°C to 8°C or −70°C ± 10°C before testing. Samples held at 2°C to 8°C were tested for PRRSV antibodies at Boehringer Ingelheim Vetmedica Inc, Health Management Center, Ames, Iowa, USA (BIVI-Ames) using a commercially available ELISA kit (IDEXX Laboratories, Westbrook, Maine, USA). Results were reported as negative (ELISA S/P ratio of < 0.4) or positive (ELISA S/P ratio ≥ 0.4). Samples held at −70°C ± 10°C were tested for PRRSV ribonucleic acid (RNA) by quantitative real-time polymerase chain reaction (qPCR; bioScreen GmbH, Münster, Germany). Results were reported as genome equivalent/mL (log10 GE/mL).

**Vaccination**

Gilts in both Experiment 1 and Experiment 2 were administered either the test vaccine or the placebo at approximately 90 ± 3 d of gestation and at 8 mo of age, respectively. In Experiment 1, gilts were vaccinated intramuscularly (IM) on the right side of the neck on day 0 and a repeat injection was administered on the left side of the neck on day 14. Gilts in group 1A were administered the placebo [phosphate-buffered saline (PBS)] and gilts in group 1B received 10× overdose [approximately 1 × 108 50% Tissue Culture Infective Dose (TCID)50/dose] of the test vaccine (ReproCyc PRRS EU; Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA). All gilts in Experiment 2 were vaccinated IM once on the right side of the neck. Specifically, gilts in group 2B (low titer) and 2C (high titer) were administered 2 mL of the test vaccine (ReproCyc PRRS EU; Boehringer Ingelheim Vetmedica) at the anticipated minimum immunizing dose (MID) of 1 × 104.43 (TCID)50/dose and at 1 × 103.90 TCID50/dose, respectively, while gilts in groups 2A and 2D received 2 mL of the placebo product (placebo matched product without PRRS 94881 MLV).

**Post-vaccination observations**

Gilts in Experiment 1 were observed once daily for clinical signs of disease including behavior (normal, recumbent, shivering, lethargic, or unconscious), respiration (normal, mild coughing, severe coughing, sneezing, abdominal breathing, or rapid respiration), digestion (normal, vomiting, diarrhea, and reduced or no appetite), and other (normal, hernia, thin, lame, edema around the eyes, etc.) on day −1 to their respective DOF + 21. Similarly, gilts in Experiment 2 were observed once daily for clinical signs of disease from day −1 to 21 and at least 3 times weekly from day 22 to 115.

**Injection site observations**

For gilts in Experiment 1, all injection sites were examined for redness (none, slight, moderate, or severe), swelling (none, minimal, slight, moderate, or severe), heat, by feel, (normal, warm, or hot), and pain (absent or present during palpation) just before vaccination and at 1 and 4 h post-vaccination on day 0 and once daily from day 1 through day 14. All injection sites on the left side of the neck were monitored for signs of local reactions just before vaccination and at 1 and 4 h post-vaccination on day 14 and once daily from day 15 through day 28. Additionally, any gilt that exhibited an injection site reaction on the right and/or left side of the neck was observed until the reaction resolved or the end of the study 21 d post-farrow (DOF + 21).

**Estrus synchronization and breeding**

Beginning on day 8 and continuing through day 21, all gilts in Experiment 2 were administered 6.8 mL of altrenogest (Matrix; Merck Animal Health, Whitehouse Station, New Jersey, USA) once daily on the feed for estrus synchronization purposes. On day 26 to 32, gilts were subsequently observed for estrus and were artificially inseminated a minimum of 2 times using semen obtained from a PRRS-negative boar farm upon determination of standing receptivity. Gilts were checked for pregnancy by ultrasound on day 84, approximately 55 ± 3 d after breeding.

**Animals removed from the trial**

No animals were removed from Experiment 1, while 41 gilts were excluded from Experiment 2 before challenge inoculation. Specifically, 5, 2, 3, and 1 gilt(s) from groups 2A, 2B, 2C, and 2D, respectively, did not display estrus after synchronization, were not bred, and were removed from the trial. After pregnancy was determined, 16 gilts (2 from group 2A, 9 from group 2B, 4 from group 2C, and 1 from group 2D) were removed from the trial by day 89 as a result of lameness, not being pregnant, or late breeding. Additionally, 5, 1, and 5 gilts from groups 2A, 2B, and 2C, respectively, were randomly selected for removal by day 104, so that there was a total of 16 bred gilts in each of the challenged groups. Similarly, 3 gilts from group 2D were randomly selected for removal, so that the negative control group consisted of 5 bred gilts.

**Challenge inoculation**

On day 118, gilts in groups 2A, 2B, and 2C were challenged intranasally (IN) with 4 mL (2 mL per nare) and 1 mL with 2 mL of a heterologous Type-I PRRSV. Briefly, on the day of the challenge, the challenge virus was thawed and diluted with minimum essential medium (MEM) to a targeted titer of 1 × 106 TCID50/mL dose. The challenge virus was isolated from an 8-week-old pig on a German farm with lungs infected by Type-I PRRS that had previously shown signs of respiratory distress. Postmortem findings confirmed interstitial pneumonia and lung lesions suggestive of PRRSV. The challenge isolate was directly propagated on MA104 cells and a culture stock containing only Type-I PRRSV was developed for future challenge studies. Pre- and post-challenge titers were carried out and the challenge material was determined to have a mean titer of 1 × 106.30 TCID50/mL dose.

**Post-challenge observations**

Gilts in Experiment 2 were observed once daily for clinical signs of disease on study days 116 through DOF + 20. Gilts were visually examined in crates and scored for clinical signs including respiration (0 = normal, 1 = panting/rapid respiration, 2 = dyspnea, or 3 = dead), behavior (0 = normal, 1 = mild-to-moderate lethargy, 2 = severely
lethargic or recumbent, or 3 = dead), and cough (0 = none, 1 = soft or intermittent, 2 = harsh or severe, repetitive, or 3 = dead). Observing personnel were blinded from treatment group assignment.

Rectal temperatures
Rectal temperatures (°C) were collected from gilts in Experiment 1 once daily using a self-calibrating digital thermometer on days −1, 1 to 13, and 15 to 28. On vaccination days (days 0 and 14), rectal temperatures were recorded just before vaccination, as well as 4 h post-vaccination.

Farrowing
Gilts in Experiment 1 farrowed on day 22 to 30, while gilts in Experiment 2 farrowed on days 134 to 147. On the day of parturition, gilts in both experiments were administered oxytocin (Vet Tek, Blue Springs, Missouri, USA), according to label directions, to assist with farrowing as needed. On the day of birth, all live piglets in Experiments 1 and 2 were administered 1.0 mL of an iron injection (either Durvet, Blue Springs, Missouri, USA or Phoenix Scientific, St. Joseph, Missouri, USA) IM in the right ham to prevent iron deficiency anemia, as well as gentamicin (Sparhawk Laboratories, Lenexa, Kansas, USA), according to label directions, to prevent scours.

Reproductive performance of gilts
Farrowing was data was recorded for all gilts in Experiments 1 and 2. On each gilt's DOF (defined as the day the first piglet was delivered), all piglets were classified into 1 of 5 categories: mummy, stillborn, weak, healthy, or crushed/mortality. A live piglet at birth was defined as any piglet that was healthy, weak, or crushed/mortality. The number of surviving piglets was determined on the last day of the trial (DOF + 21 for Experiment 1 and DOF + 20 for Experiment 2).

Observations of piglets
All live piglets in both experiments were observed once daily for clinical signs beginning on DOF + 1 and continuing through to the end of the trial (DOF + 1 to DOF + 21 for Experiment 1 and DOF + 1 to DOF + 20 for Experiment 2). A daily total clinical observation score was determined as a summation of respiration, behavior, and cough scores, as previously described (post-challenge observations for gilt in Experiment 2).

Piglet average daily weight gain
Individual body weights (kg) of all piglets were collected on the day of birth. Body weights were also collected on DOF + 21 and DOF + 20 for piglets in Experiment 1 and Experiment 2, respectively, or on the day a piglet was found dead. Average daily weight gain (ADWG) was determined for all surviving piglets from DOF through DOF + 21 for Experiment 1 or DOF + 20 for Experiment 2.

Postmortem examinations
All gilts in Experiment 1 were euthanized by sedation and electrocution on DOF + 21. If an injection site reaction (swelling) was still present, tissue samples were collected from the site and placed in a container with an appropriate amount of 10% formalin and submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL, Ames, Iowa, USA) for histopathologic examination (embedded in paraffin, sectioned, and stained with hematoxylin and eosin stain) by a board-certified veterinary pathologist who was blinded from animal treatments.

All surviving piglets in Experiment 1 were euthanized and necropsied on DOF + 21. The lungs were examined for gross lesions and the percent pathology for each lobe was recorded. Total lung lesion scores were determined for each pig using the European Union (EU) enzootic pneumonia formula. Specifically, total lung lesion scores were measured as a percentage of lung involvement calculated according to a weighting formula that accounts for the relative weight of each of the 7 lobes. The assessed percentage of lung lobe area with typical lesions was multiplied by the lobe factor (left apical = 0.05, left cardiac = 0.06, left diaphragmatic = 0.29, right apical = 0.11, right cardiac = 0.10, right diaphragmatic = 0.34, and intermediate = 0.05) and the total weighted lung lesion score was then determined. Similarly, lung pathology was recorded for all stillborn piglets (excluding mummies), as well as piglets that died or were euthanized before study completion.

PRRSV quantitation in lung tissue
For all surviving piglets in Experiment 1 and all piglets dead at delivery or that died or were euthanized before DOF + 21 (Experiment 1) or DOF + 20 (Experiment 2), 2 lung tissue samples were collected on DOF + 21 or the day of death. One sample was placed in a Whirl-Pak bag (US Plastic Corporation, Lima, Ohio, USA) and the other in a container with 10% formalin. Samples in Whirl-Pak bags were stored at −70°C ± 10°C and then shipped to bioScreen GmbH for viremia testing for PRRSV RNA by qPCR as described by Revilla-Fernandez et al (27). Specifically, the 2′× TaqMan Universal PCR Kit (Applied Biosystems, Foster City, California, USA) with AmpErase UNG (Applied Biosystems), the EU6-MGB: CTGGTGAAGACCCGGGAC probe, and the primers EU6-343f-plus: GTRGAAAGTGCTGCAGGYCTCCA (sense) and EU6-462r-plus: CACGAGGCTCCGAGYCCW (antisense) were used. Results were reported as log_{10} GE/mL for left and right/intermediate lung samples. Formalin-fixed samples were stored at room temperature for approximately 1 wk before being submitted to ISU VDL for embedding in paraffin blocks and subsequent storage, again at room temperature, for possible future testing.

Statistical analyses
The statistical analyses and data summaries were done using SAS software, Version 8.2 (SAS Institute, Cary, North Carolina, USA). All data for both experiments were summarized descriptively (n = sample number, minimum, maximum, mean, median, interquartile range, or confidence interval) based on the type of variable. All data were analyzed assuming a completely random design structure and tests on differences were designed as 2-sided tests at α = 0.05, with differences considered significant if P ≤ 0.05.

The main objective of Experiment 1 was to compare the vaccinated group (group 1B) with the non-vaccinated group (group 1A), with primary variables of gilt reproductive performance and survival rate of offspring at weaning (DOF + 21) and secondary, supportive parameters of systemic post-vaccination reactions, local injection site reactions, placental infection of piglets, and general health of
offspring during the suckling period (clinical observations, ADWG, and lung lesions).

Specifically, gilt reproductive performance and the survival rate of offspring, as well as gilt rectal temperatures, mean duration of injection site reactions, transplacental infection of piglets, and live piglets plus mummies and stillborn piglets that were PRRSV-positive by qPCR, viremia in piglets per litter, and piglet lung lesions, were analyzed using the Wilcoxon-Mann-Whitney test. Clinical observations of gilts, proportion of gilts with an increase in rectal temperature $1.5^\circ$C compared to baseline days, proportion of gilts with an injection site reaction for at least 1 d, viremia post-vaccination in gilts, post-vaccination serology of gilts, and proportion of piglets per litter with a positive clinical observation score for at least 1 d were evaluated using Fisher’s exact test. Finally, mean daily rectal temperature of gilts, mean piglet body weight per litter on DOF, and mean piglet ADWG per litter from DOF through DOF + 21 were compared using the analysis of variance (ANOVA) procedure.

The main objective of Experiment 2 was to compare the 2 vaccinated groups (groups 2B and 2C) to the unvaccinated challenge control group (group 2A). Animals removed from the trial were included in their respective parameter of analysis until removal. Primary variables included proportions of live piglets on day of farrowing (DOF) and proportions of live piglets at 20 d of age (DOF + 20), with supportive variables consisting of clinical observations post-vaccination and post-challenge, serology, viremia, and reproductive performance of the gilts, as well as viremia, clinical observations, and ADWG for the piglets.

Specifically, frequency tables were generated of gilts with at least 1 positive finding for clinical observations post-vaccination and post-challenge, for positive ELISA results, and for positive qPCR (qualitative analysis) of gilt viremia and differences between the challenge control and vaccine groups were tested using Fisher’s exact test. Gilt viremia data were also evaluated (quantitative analysis) separately for each day and positive versus negative (assigned values of 3.0 and 0.0 log$_{10}$ GE/mL, respectively) were compared using the Wilcoxon-Mann-Whitney test. Differences in gilt reproductive performance between groups were also tested by the Wilcoxon-Mann-Whitney test. Piglet viremia data were evaluated qualitatively (calculated percentages of positive piglets per litter used as single values for comparison of groups) and quantitatively (calculated median qPCR values per litter used as single values for comparison of groups) for separate comparisons for each day using the Wilcoxon-Mann-Whitney test. Individual piglet qPCR data were used for summary statistics and viral load in lung samples from dead piglets before DOF + 20 were evaluated descriptively only. Individual piglet ADWGs were calculated for between DOF and DOF + 20 and differences among treatment groups were analyzed using the ANOVA procedure and subsequent t-tests. Least squares means of groups and differences between LS means with 95% confidence intervals

![Gilt mean rectal temperatures](image.png)
The Canadian Journal of Veterinary Research

Post-vaccination observations

In Experiment 1, no gilt mortalities occurred in either group 1A or 1B (placebo-vaccinated or repeat 10× overdose, respectively). From day 0 + 4 h to DOF + 21, 50% of gilts in group 1A and 75% of gilts in group 1B exhibited at least 1 abnormal clinical finding for a minimum of 1 d (difference between groups was not significant; \( P = 0.6084 \)). Specifically, 4 gilts in group 1A and 6 gilts in group 1B exhibited abnormal clinical findings. Two gilts had reduced or no appetite for 2 d and 1 gilt was lethargic for 1 d; 1 gilt had reduced or no appetite for 2 d and was lethargic for 2 consecutive d; 1 gilt had rapid respiration, reduced or no appetite for 1 d each, and was lethargic on 2 consecutive days; and 1 gilt had a small abscess on the right side of the neck (noted before vaccination) for 28 d and other signs (no description other than farrowing) on 1 d. No analysis was conducted on gilts in Experiment 2 as no abnormalities were noted after vaccination.

Results

Table I. Group summary of number of live piglets per litter at weaning. No significant differences were noted between groups 1A and 1B, while groups 2B and 2C had significantly higher numbers of live piglets at weaning than group 2A (\( P = 0.0063 \) and 0.0026, respectively)

<table>
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<th>Mean</th>
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\( a \) DOF + 21.
\( b \) DOF + 20.
\( c \) Compared to group 2A.
DOF — Day of farrowing; N/A — Not applicable.

Figure 2. Summary of group reproductive performance results (number of piglets per litter) on day of farrowing (DOF) and summary by group of mean percentage of piglets per litter in each category at farrowing. There were significant differences between groups 2B and 2A for mean percentage of live, healthy live, and mummified piglets at birth (\( P = 0.0184 \), \( P = 0.0138 \), and \( P = 0.0190 \), respectively) and between groups 2C and 2A for mean percentage of live, healthy live, weak live, and mummified piglets.

\* Significant difference among groups compared to group 2A.
\** Negative control group; no analysis conducted.

Table I. Group summary of number of live piglets per litter at weaning. No significant differences were noted between groups 1A and 1B, while groups 2B and 2C had significantly higher numbers of live piglets at weaning than group 2A (\( P = 0.0063 \) and 0.0026, respectively)

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<td>0.0026c</td>
</tr>
<tr>
<td>2Db</td>
<td>9</td>
<td>14</td>
<td>10.0</td>
<td>10.8</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\( a \) DOF + 21.
\( b \) DOF + 20.
\( c \) Compared to group 2A.
DOF — Day of farrowing; N/A — Not applicable.
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In Experiment 1, 1 gilt from each group exhibited an injection site reaction for at least 1 d until day 14 ($P = 1.0000$), whereas no gilts in group 1A, but 7 out of 8 gilts in group 1B exhibited an injection site reaction of either redness, heat, and/or pain for at least 1 d from day 14 onwards ($P = 0.0014$). From day 0 + 1 h to day 14, gilts in groups 1A and 1B had median injection site reaction duration of 0.0 and 2.0 d, respectively. Swelling was measurable for only 1 gilt in group 1B (slight swelling on the right side on day 1 to day 4 and on day 17 and moderate swelling on the right side on days 18 to 23 and days 41 to 43), which was later determined to be an abscess. Swelling of the 1 gilt in group 1A (day 0 + 4 to day 1) and the 6 gilts in group 1B was found to be minimal.

**Injection site observations**

In Experiment 2, percentages of total abnormal findings (a clinical score $> 0$ for respiration, behavior, and/or cough indicating clinical disease) for at least 1 d for gilts in groups 2A (placebo-vaccinated and challenged), 2B [low-titer (MID) vaccinated and challenged], 2C (high-titer vaccinated and challenged), and 2D (placebo-vaccinated and not challenged) were 25.0%, 25.0%, 38.0%, and 60.0% from day 116 (2 d before challenge) to DOF + 20, respectively. No significant differences in frequency of gilts positive for clinical disease were detected among vaccinated groups and the challenge control group during this time period ($P \geq 0.7043$).

**Rectal temperatures**

In Experiment 1, mean rectal temperature for gilts was within the normal limits throughout the study and ranged from 37.9°C to 38.8°C in group 1A and from 38.0°C to 39.2°C in group 1B (Figure 1).

**Reproductive performance of gilts**

No significant differences were found between groups 1A and 1B for median percentages of live piglets (healthy + weak + crushed/mortality) per litter at farrowing (89.0% and 91.7%, respectively; $P = 1.0000$). Median percentages of healthy live piglets per litter (83.8% and 89.3%, respectively; $P = 0.9262$) and median percentages of weak live, stillborn, mummified, and crushed/mortality piglets per litter were 0.0% for all parameters for both groups ($P \geq 0.8825$). There was no significant difference between groups for median percentages of live piglets at the time of weaning (100.0% and 90.5% for groups 1A and 1B, respectively; $P = 0.2103$).

Groups 2B and 2C had significantly higher percentages of live and healthy live piglets per litter ($P \leq 0.0455$) than group 2A. Group 2C had significantly lower percentages of weak live piglets per litter ($P = 0.0024$), groups 2B and 2C had significantly lower percentages of mummies per litter ($P \leq 0.1965$), and there were no significant differences among groups for the percentages of stillborn or crushed/mortality piglets per litter ($P \geq 0.1965$). Median percentages of live piglets per litter at weaning were significantly higher in groups 2B and 2C than in group 2A ($P = 0.0203$ and $P = 0.0022$, respectively). The number of piglets born in each category per group is summarized in Figure 2, while the number of piglets weaned per group is shown in Table I.

**Piglet clinical observations**

There was no significant difference between groups 1A and 1B for median percentages of piglets per litter that were positive for clinical disease for at least 1 d (8.7% and 10.6%, respectively; $P = 0.9814$). In Experiment 2, median percentages of piglets per litter that were positive for clinical disease for at least 1 d were significantly lower in groups 2B and 2C than in group 2A (25.0% and 25.0% versus 100.0%, respectively; $P \leq 0.0001$).

**Average daily weight gain**

For piglets in both Experiments 1 and 2, there were no significant differences in body weight among groups on DOF ($P = 0.6766$ and $P \geq 0.2972$, respectively). Specifically, least squares mean body weights for piglets in groups 1A and 1B were 1.55 and 1.50 kg, respectively, and the mean body weight for piglets in groups 2A, 2B, 2C, and 2D were 1.34, 1.43, 1.40, and 1.39 kg, respectively. No significant difference in least squares means for ADWG was detected between groups 1A and 1B from DOF to DOF + 21 (222 g/d and 226 g/d, respectively; $P = 0.8760$). Conversely, piglets in groups 2B and 2C had significantly higher least squares mean ADWG than those in group 2A from DOF to DOF + 20 (194 g/d and 197 g/d versus 130 g/d, respectively; $P = 0.0028$) when weight on DOF was used as a covariate for analysis (Figure 3).

**PRRSV viremia testing**

No PRRSV RNA was detected in the serum of any gilt in either experiment on day 0. In Experiment 1, all gilts in group 1A remained negative for the duration of the experiment. After vaccination (day 0), 2 out of 8 (25%) of gilts in group 1B were positive for PRRSV RNA by qPCR on day 14 (difference not significant; $P = 0.4667$), whereas no gilts in either group were positive on DOF and DOF + 21. In Experiment 2, all gilts in both groups 2A and 2D remained negative until the day of challenge and for the duration of the experiment, except for 1 gilt in group 2D that was positive on DOF + 7 (tested negative at all other time points).

After vaccination (day 0), 50% of gilts in groups 2B and 2C were PRRSV RNA positive, whereas 36% were PRRSV RNA positive on
From days 14 to 56, only 4% of gilts in group 2B remained positive, while up to 4% of gilts in group 2C were intermittently qPCR positive. All gilts were qPCR negative for PRRSV RNA on day 84 and day 118 (day of challenge). After the challenge, groups 2B and/or 2C had significantly lower percentages of positive gilts than group 2A on day 125 (7 days post challenge; DPC) (31.0% and 25.0% versus 100.0%, respectively; \( P < 0.0001 \)), day 132 (14 DPC) (19.0% (\( P = 0.0290 \)) and 31.0% (\( P = 0.1556 \)) versus 63.0, respectively), and day 137 (0.0% and 6.0% versus 94.0%, respectively; \( P < 0.0155 \)). No significant differences in percentages of positive gilts were detected between the vaccinated groups and the challenge control group on day 137 and day 140 (\( P > 0.1719 \)) (Table II).

Conversely, significant differences in median percentages of piglets positive for PRRSV RNA were found on multiple occasions when all piglets (alive or dead) born to gilts in Experiment 2 were evaluated. Specifically, groups 2B and 2C had significantly lower median percentages of positive piglets than group 2A (\( P = 0.0381 \) and \( P = 0.0018 \), respectively) on day 118 (day of challenge). On day 125, groups 2B and 2C had significantly lower median percentages of positive piglets than group 2A (\( P = 0.0230 \)) compared to group 2A.

No significant differences between groups in Experiment 1 were found in median percentages of piglets that were positive for PRRSV RNA on the day of farrowing. Specifically, median percentages of zero of precolostral blood samples from live piglets per litter from groups 1A and 1B were qPCR positive for PRRSV RNA on day 7 (day of challenge). After the challenge, groups 2B and/or 2C had significantly lower percentages of positive piglets than group 2A on day 125 (7 days post challenge; DPC) (31.0% and 25.0% versus 100.0%, respectively; \( P < 0.0001 \)), day 132 (14 DPC) (19.0% (\( P = 0.0290 \)) and 31.0% (\( P = 0.1556 \)) versus 63.0, respectively), and day 137 (0.0% and 6.0% versus 94.0%, respectively; \( P < 0.0155 \)).

No significant differences in percentages of positive piglets were detected between the vaccinated groups and the challenge control group on day 137 and day 140 (\( P > 0.1719 \)) (Figure 4).

No significant differences between groups in Experiment 1 were found in median percentages of piglets that were positive for PRRSV RNA on the day of farrowing. Specifically, median percentages of zero of precolostral blood samples from live piglets per litter from groups 1A and 1B were qPCR positive for PRRSV RNA on day 7 (day of challenge). After the challenge, groups 2B and/or 2C had significantly lower percentages of positive piglets than group 2A on day 125 (7 days post challenge; DPC) (31.0% and 25.0% versus 100.0%, respectively; \( P < 0.0001 \)), day 132 (14 DPC) (19.0% (\( P = 0.0290 \)) and 31.0% (\( P = 0.1556 \)) versus 63.0, respectively), and day 137 (0.0% and 6.0% versus 94.0%, respectively; \( P < 0.0155 \)).

PRRSV serologic testing

All gilts in Experiment 1 were seronegative for PRRSV by ELISA testing on day 0. After vaccination, 75% of gilts in group 1B were shown to have seroconverted by day 14 and 100% by DOF. Gilts in group 1A remained seronegative throughout the study. In Experiment 2, all gilts were seronegative for PRRSV by ELISA testing on day 0 and day 7. All gilts in groups 2A and 2D remained seronegative up to and including the day of challenge (day 118) and until the conclusion of the trial (DOF + 20), respectively. Conversely, on day 14, the percentage of PRRSV ELISA positive gilts in group 2B was 18% and in group 2C was 21%, which increased to 65.0% in 2B and 60.0% in 2C on day 56. On the day of challenge (day 118), percentages of seropositive gilts in groups 2B (56.0%) and 2C (50.0%) were decreasing. On day 125, percentages of seropositive
gilt in group 2A died after blood collection and death was confirmed at necropsy to be due to a lacerated jugular vein and secondary blood loss. In Experiment 1, all piglets in group 1A that were necropsied on DOF + 21 had no lung pathology in any lobe, whereas 3 out of 68 piglets in group 1B had minor lung lobe pathology (total lung lesion scores of ≤ 1.85%; lesions described as very small areas of lung consolidation likely due to a past bacterial infection) and 1 piglet had 100% pathology in each lung lobe (total lesion score of 100%; lesions described as pneumonia and pleuritis likely due to a bacterial infection). There were no significant differences among groups for median percentages of piglets per litter that were positive for PRRSV RNA in the lung tissues by qPCR (0.0% and 10.0%, respectively; \( P = 0.0769 \)). In Experiment 2, of the piglets that were either born dead, died, or were euthanized before DOF + 20, the mean lung qPCR results were 4.676, 4.092, 3.547, and 0.000 \( \log_{10} \) GE/mL for groups 2A, 2B, 2C, and 2D, respectively (Table IV) (no statistical analysis conducted).

### Table II. Group summary of percentages of piglets that were positive for PRRSV RNA as determined by qPCR. Groups 2B and 2C had significantly lower mean percentages of positive piglets than group 2A on day of farrowing (DOF) and DOF + 7 (\( P = 0.0381 \) and \( P = 0.0175 \), respectively), while on DOF + 13, group 2B had significantly lower percentages of positive piglets than group 2A (\( P = 0.0216 \)).

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>Mean</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOF</td>
<td>2A</td>
<td>86.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>58.1</td>
<td>0.0381( ^a )</td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td>55.0</td>
<td>0.0018( ^a )</td>
</tr>
<tr>
<td></td>
<td>2D</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>DOF + 7</td>
<td>2A</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>76.6</td>
<td>0.0293( ^a )</td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td>78.6</td>
<td>0.0175( ^a )</td>
</tr>
<tr>
<td></td>
<td>2D</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>DOF + 13</td>
<td>2A</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>75.4</td>
<td>0.0216( ^a )</td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td>84.0</td>
<td>0.0860( ^a )</td>
</tr>
<tr>
<td></td>
<td>2D</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>DOF + 20</td>
<td>2A</td>
<td>90.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>75.3</td>
<td>0.0614( ^a )</td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td>81.6</td>
<td>0.183( ^a )</td>
</tr>
<tr>
<td></td>
<td>2D</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Compared to group 2A.

DOF — Day of farrowing.

### PRRSV quantitation in lung tissue

There was no significant difference between groups 1A and 1B in median percentages of piglets per litter that were positive for PRRSV RNA in the lung tissues by qPCR (0.0% and 10.0%, respectively; \( P = 0.0769 \)). In Experiment 2, of the piglets that were either born dead, died, or were euthanized before DOF + 20, the mean lung qPCR results were 4.676, 4.092, 3.547, and 0.000 \( \log_{10} \) GE/mL for groups 2A, 2B, 2C, and 2D, respectively (Table IV) (no statistical analysis conducted).

### Discussion

Vaccinating gilts and sows with a PRRS MLV vaccine is beneficial for several reasons, including increased farrowing and weaning rates and decreased number of premature farrowings (1,4,7,8). Vaccination of dams also has positive effects on the piglets, including increased ADWG and decreased clinical signs (12). The effectiveness of a PRRS MLV vaccine in improving reproductive performance is vital because loss from piglet deaths, for whatever reason (mummified, stillborn, and/or disease-related), results in fewer piglets weaned and therefore dramatically affects the economic productivity of the herd. A PRRS MLV vaccine must be safe to administer, however, in that it does not cause reproductive failure, local or systemic reactions, and negatively affect piglet survivability and growth performance.

In Experiment 1, primary criteria for evaluating safety included reproductive performance at farrowing and survival rate at weaning, with supportive parameters of local and/or systemic reactions to vaccination and transplacental infection, as well as general health of piglets (clinical observations and growth performance) during the suckling period.

In Experiment 2, effectiveness was primarily determined by evaluating reproductive performance at farrowing (number of live-born piglets) and the number of piglets at weaning. This was supported by evaluating gilts for clinical assessments post-vaccination and post-challenge, PRRSV serology, and viremia, as well as evaluation for total number of piglets, healthy live piglets, weak live piglets, mummies, stillborn piglets, and crushed/mortality piglets born per litter. Piglets were assessed for viremia, clinical observations, and ADWG. According to these criteria, the present data clearly demonstrated that vaccination of gilts with the novel PRRSV strain 94881 MLV vaccine (ReproCyc PRRS EU; Boehringer Ingelheim Vetmedica) is a safe option for preventing reproductive losses associated with the PRRS virus.

Specifically, PRRS-naïve gilts that were given a repeat 10× overdose did not exhibit any relevant differences in median percentages of live piglets born or live piglets at weaning compared to non-vaccinated placebo controls. During the suckling period, there were no significant differences between the vaccinated and non-vaccinated groups for systemic reactions to vaccination (post-vaccination observations), gilt viremia, and general health of piglets (clinical observations, least squares mean body weight at birth, and ADWG). There were no significant differences between groups for injection site observations after the initial 10× overdose and no biologically relevant differences in rectal temperatures after vaccination (did not exceed 40.0°C).
In Experiment 2, the vaccine also met the necessary requirements for efficacy as significantly higher percentages of live pigs were born and weaned in the vaccinated group than in the non-vaccinated challenge control group. There were significant beneficial differences in 1 or both vaccinated groups compared to the challenge control group for percentages of healthy live piglets, weak live piglets, and mummified piglets born per litter. Results of gilt post-vaccination clinical observation were in alignment with the safety study, as there were no abnormal assessments for any group from day 1 through day 21 and no significant difference among groups for abnormal clinical assessments for at least 1 d from day 1 through day 113.

Similarly, there were no significant differences in gilt clinical observations post-challenge between the low-titer and high-titer vaccinated groups compared to the placebo-vaccinated and challenged group. Interestingly enough, however, a higher percentage of gilts in the negative control group (placebo-vaccinated and not challenged) exhibited a clinical observation for at least 1 d from day 116 through day 113 even though a 10^3 overdose of the vaccine had been administered. Vaccinated gilts were also shown to have seroconverted as early as 14 d post-vaccination. Vaccinated gilts experienced a brief period of viremia after treatment was administered, as evidenced by positive gilts on day 7. Differences in viremia between vaccinated groups (groups 2B and 2C) were not significant from days 14 through 56, however, and all gilts were negative on day 84 and day 118.

Conversely, there were significantly lower percentages of qPCR-positive gilts in the vaccinated groups than in the challenge control group after the challenge [on days 125, 132 (low dose only), DOF, and DOF + 13]. Piglets born to vaccinated gilts also exhibited favorable results compared to piglets born to challenge control gilts, as 1 or both vaccinated groups had significantly lower percentages of piglets per litter that were positive for abnormal clinical findings for at least 1 d from DOF + 1 through DOF + 20, significantly higher ADWG, and significantly lower percentages of piglets per litter that were viremic on DOF, DOF + 7, and DOF + 13. These results clearly indicate that even a 10^3 overdose of the vaccine was safe to administer during gestation and effectively prevented the detrimental effects of PRRSV infection in both gilts and piglets born to vaccinated gilts.

While a number of studies have been published describing the safety of NA-type PRRS modified live virus (MLV) vaccines in bred females, less information is available about the safety of EU-type MLV vaccines. One trial examined the safety of a single dose of a commercially available EU-type MLV PRRSV vaccine in gilts and sows that were in either early or late pregnancy and in lactating sows (7). In the trial, safety was established according to the lack of significant differences in local or systemic reactions between vaccinated and non-vaccinated animals. Additionally, fluid and/or tissue samples collected from aborted piglets were negative for PRRSV RNA. These results are similar to the present safety evaluation data, as vaccinates exhibited neither significantly increased incidence of local or systemic reactions after the first vaccination and there were no significant differences between groups for gilt viremia on DOF, even though a 10^3 overdose of the vaccine had been administered.

A number of studies have examined the effects of the use of EU-type MLV vaccines in endemic situations. The effects of an EU-type MLV PRRS vaccine were evaluated in lactating sows and pregnant gilts and sows on 3 Polish farms with histories of chronic PRRSV infection (7). The vaccine was found to be beneficial as live piglets born and piglets weaned increased significantly compared to pre-vaccination data for the same animals.

Table III. Summary of percentages of piglets per litter with lung lesions and total lung lesion scores per group. There were no significant differences between groups 1A and 1B in either category

<table>
<thead>
<tr>
<th>Piglet lung lesions</th>
<th>Group</th>
<th>n</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of piglets per litter</td>
<td>1A</td>
<td>8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.00</td>
<td>0.2000</td>
</tr>
<tr>
<td></td>
<td>1B</td>
<td>8</td>
<td>0.0</td>
<td>16.7</td>
<td>4.89</td>
<td></td>
</tr>
<tr>
<td>Total lung lesion scores</td>
<td>1A</td>
<td>72</td>
<td>0.0</td>
<td>0.0</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1B</td>
<td>68</td>
<td>0.0</td>
<td>100.0</td>
<td>1.52</td>
<td>0.0531</td>
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</tbody>
</table>

Table IV. Summary of qPCR results (log_{10} GE/mL) for PRRSV in lungs of piglets by group. In Experiment 2, although no statistical analysis was conducted to compare qPCR results of piglets that were born dead or died before day of farrowing (DOF) + 20, the median and mean lung qPCR results were lower in dead piglets in groups 2B and 2C than those in group 2A

<table>
<thead>
<tr>
<th>Piglet lung qPCR</th>
<th>Group</th>
<th>N</th>
<th>Min</th>
<th>Max</th>
<th>Median</th>
<th>95% CI</th>
<th>Q Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2A</td>
<td>141</td>
<td>0.00</td>
<td>7.95</td>
<td>5.140</td>
<td>4.810</td>
<td>4.260</td>
<td>4.676</td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td>79</td>
<td>0.00</td>
<td>7.45</td>
<td>4.780</td>
<td>3.000</td>
<td>2.620</td>
<td>4.092</td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td>75</td>
<td>0.00</td>
<td>6.84</td>
<td>4.220</td>
<td>3.000</td>
<td>5.100</td>
<td>4.676</td>
</tr>
<tr>
<td></td>
<td>2D</td>
<td>4</td>
<td>0.00</td>
<td>0.00</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

qPCR — Quantitative real-time polymerase chain reaction.
In a study conducted on an endemically PRRSV-infected farrow-to-finish farm in Greece, a group of gilts and sows were administered a single dose of an EU-type MLV vaccine at approximately 6 mo of age and 10 d after farrowing, respectively, while an additional group of gilts and sows were left untreated (1). The vaccinated group had significantly improved farrowing rates, higher numbers of live-born piglets, fewer dead or mummified piglets, and more weaned pigs (average of 0.7 piglets per litter) than the non-vaccinated group.

Another study described significantly improved farrowing rates, including higher numbers of live-born and fewer numbers of mummified and stillborn piglets per litter, when pigs on PRRSV-positive farms in Thailand were treated with either an EU-type or NA-type PRRSV MLV vaccine (4). Unfortunately, the data were reported as a generalization of vaccinates versus non-vaccinates (no groups based on timing of vaccination or type of PRRSV vaccination received). The authors did note, however, that vaccination improved fertility rate in gilts by a greater percentage (7.3%) than in sows (6.7%, 3.8%, 2.0%, and 0.4% in parity numbers 1, 2 to 3, 4 to 5, and ≥ 6, respectively).

While results similar to the present data [significantly improved reproductive performance at farrowing (more live and healthy live piglets born and fewer weak live piglets and mummies born) and more live pigs at weaning (average of 3.65 piglets per litter)] have been reported, these studies contrast directly with Experiment 2 as they did not involve an administered challenge of known virulence at a time of high susceptibility, the serological status of each animal before vaccination was not known, and the PRRSV strains present at the trial locations were not identified.

Only 1 study was found that examined the efficacy of 2 EU-type MLV PRRSV vaccines in gilts that also involved an administered challenge of PRRS virus. Using gilts vaccinated (IM) once at 24 d before AI and subsequently challenged with a heterologous strain, Scortti et al evaluated the effects of 2 different EU-type MLV PRRSV vaccines on gilts and their progeny (8). While no adverse reactions to either vaccine were noted, all vaccinated gilts except 1 developed vaccine-induced viremia. Neither vaccine provided complete protection against challenge-induced viremia as the virus was isolated in the serum of approximately 40% of all vaccinated gilts. Conversely, reproductive performance significantly improved in both groups of vaccinated gilts, with more live piglets and fewer stillborn and mummified piglets born, and decreased incidence of transplacental infection of piglets after the challenge. Additionally, ADWG and piglet mortality rates were not significantly affected by congenital infection. While the reproductive results in this study are similar to the data presented here, only 50.0% of vaccinated gilts in group 2B and 36.0% of vaccinated gilts in group 2C became viremic as a result of vaccination. The majority of vaccinated gilts (69.0% of group 2B and 75.0% of 2C) were protected from challenge-induced viremia and, while congenital infection was noted for piglets born to vaccinated gilts, piglet growth and survival rates were significantly improved by vaccination.

The data presented here has shown that this novel vaccine strain provides gilts with far better protection against PRRSV during pregnancy than inactivated vaccines. Inactivated EU-type vaccines have been shown to be protective in the face of a homologous PRRSV challenge, significantly increasing percentages of live and healthy piglets born (18) and decreasing duration of viremia and occurrence of congenital infection, reducing percentage of mummified fetuses, and improving fetal survival (16). When a heterologous challenge was used, however, an inactivated EU-type vaccine failed to significantly protect gilts from clinical signs and viremia and decreased reproductive performance and transplacental infection (17).

These cited studies support the fact that the heterogeneity of the PRRS virus continues to make it difficult to develop a single-strain vaccine virus that is effective against the various strains of PRRSV in the field. It is difficult to directly compare the safety and efficacy of EU-type PRRS MLV vaccines in breeding females, due to the limited nature of available data. Vaccination with a PRRS MLV vaccine, however, has generally been found to positively affect reproductive performance when used in PRRSV endemic situations. The present study provides evidence that a vaccine based on the novel PRRSV MLV strain 94881 is a safe and effective way to protect against the negative clinical and economic effects of PRRS viral infection when administered to gilts before breeding.

When the novel PRRS 94881 MLV vaccine, ReproCyc PRRS EU-type, was administered as a repeat 10× overdose (approximately 1 × 10⁶ TCID₅₀/₆.0 mL) to bred gilts at approximately 90 d of gestation, there were no relevant differences for piglets in live-born, healthy live-born, general health during the suckling period, ADWG, live piglets at weaning, lung pathology, and viral load in lung tissue, and for gilts, in systemic reactions to vaccination and viremia. When a single dose of the test vaccine at various titer formulations was administered to breeding-age gilts 26 to 32 d before breeding, vaccinated gilts had more live born, healthy live-born, and weaned piglets per litter. The piglets had higher ADWG, fewer weak live and mummified piglets per litter, fewer piglets with abnormal clinical signs, fewer incidences of piglet viremia, no statistically relevant abnormal clinical assessments post-vaccination, positive serological responses as early as 14 d post-vaccination, and lower incidence of viremia following challenge.

In conclusion, A vaccine formulated from the PRRSV MLV strain 94881 has therefore been proven to be a safe and effective method of protection against the detrimental effects of virulent PRRSV infection in breeding female pigs.

Acknowledgments
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Efficient construction of *Haemophilus parasuis* mutants based on natural transformation

Junxing Li, Xiufang Yuan, Lihua Xu, Lei Kang, Jun Jiang, Yicheng Wang

**Abstract**

Studies on virulence factors and pathogenicity of *Haemophilus parasuis* have long been hindered by a lack of a consistent system for genetic manipulation. In this study, competence was induced by transferring *H. parasuis* from rich medium to starvation medium media-IV (M-IV) and iscR gene deficient mutants of *H. parasuis* were generated efficiently. Transformation frequency varied from $4.1 \times 10^{-5}$ to $1.1 \times 10^{-8}$ when using circular plasmid, and increased to about 2- to 31-fold when transformed using linearized plasmid. Allele replacement occurred efficiently in 6 strains, which are transformable using both circular and linearized pTRU, but not in another 2 strains which could only be transformed using linearized plasmid. The iscR mutants were stable for at least 20 passages in vitro. *Haemophilus parasuis* strains vary extensively in natural transformation efficiency and the method established here allows for transformation of a larger spectrum of strains with an easily accessed plasmid. This provides important tools for genetic manipulation of *H. parasuis*.

**Résumé**

Les études sur les facteurs de virulence et la pathogénicité d’*Haemophilus parasuis* ont longtemps été limitées à cause de l’absence d’un système constant de manipulations génétiques. Dans la présente étude, la compétence a été induite en transférant *H. parasuis* d’un milieu de culture riche au milieu pauvre M-IV et les *H. parasuis* mutants déficients pour le gène iscR étaient générés efficacement. La fréquence de transformation variait de $4.1 \times 10^{-5}$ à $1.1 \times 10^{-8}$ lors de l’utilisation d’un plasmide circulaire, et augmentait d’un facteur variant de 2 à 31 lorsque la transformation utilisait un plasmide linéarisé. Le remplacement d’allèle est survenu efficacement chez 6 souches, qui étaient transformables en utilisant le pTRU circulaire ou linéarisé, mais pas chez 2 autres souches qui ne pouvaient être transformées qu’en utilisant le plasmide linéarisé. Les mutants iscR étaient stables pendant au moins 20 passages in vitro. Les souches d’*H. parasuis* varient énormément dans leur efficacité de transformation naturelle et la méthode développée ici permet la transformation d’un plus large spectre de souches avec un plasmide facilement accessible. Ceci fournit d’importants outils pour la manipulation génétique d’*H. parasuis*.

(Traduit par Docteur Serge Messier)

**Introduction**

*Haemophilus parasuis* is the causative agent of Glässer’s disease, characterized by fibrinous polyserositis, arthritis, and meningitis. The bacterium is Gram-negative and a member of the family *Pasteurellaceae*. It can be a commensal bacterium colonized in the upper respiratory tract of clinically normal swine or a pathogen causative agent of Glässer’s disease, characterized by fibrinous polyserositis, arthritis, and meningitis. The bacterium is Gram-negative and a member of the family *Pasteurellaceae*. It can be a commensal bacterium colonized in the upper respiratory tract of clinically normal swine or a pathogen causatif of *Haemophilus parasuis*. It can be a commensal bacterium colonized in the upper respiratory tract of clinically normal swine or a pathogen causatif of infection (1). Therefore, it is very important to differentiate between virulent and non-virulent isolates for diagnosis and control of the disease. Current research on virulence factors of *H. parasuis* is mainly based on comparison of high and low virulence strains, and an increasing number of putative virulence factors have been found (2,3). However, the function of these putative virulence factors is still to be determined due to a lack of a genetic manipulation method for construction of deletion mutant. Construction of a genetic mutant is an efficient method for determination of virulence factors. So far, 2 different systems have been developed for transformation of *H. parasuis* including electroporation and natural transformation. A modified endogenous plasmid was used for the genetic manipulation of *H. parasuis*, and 15 out of 16 strains were transformable when the plasmid was pretreated with cell-free extracts (4). Electroporation efficiencies up to $10^5$ were achieved when done with a native plasmid by growing *H. parasuis* at low temperature (5). A Tn5-based random mutagenesis method for use in *H. parasuis* was developed for preparation of mutant pools (6). However, there have been no reports of exogenous plasmid transformed into *H. parasuis* by electroporation. Hence, this method was not widely used due to difficulty in access to endogenous plasmid. A natural transformation method was developed and mutants were constructed, but only 1/11 tested strains were naturally transformable. Moreover, the presence of specific DNA uptake signal sequence (USS) is required for natural transformation of *H. parasuis*, but the effect of the secondary messenger cyclic adenosine monophosphate (cAMP) on natural transformation is controversial...
(7,8). Recently, a successive markerless mutation system in H. parasuis through natural transformation was established, but only 1 of 6 screened strains was transformable (9). Searching for a consistent method to improve transformation efficiency for generation of H. parasuis mutants is still a challenge (2).

Natural competence has been extensively studied in Haemophilus influenzae, which is also a member of the family Pasteurellaceae. When H. influenzae was transferred from rich medium to starvation medium or from aerobic conditions to anaerobic conditions, competence was developed (10,11). The addition of cAMP to exponentially growing bacteria also induces competence (12). Transferring early exponentially growing bacteria to starvation medium also induces competence in Haemophilus parainfluenzae, Actinobacillus pleuropneumoniae, and Gallibacterium anatis (13–15).

The objective of this study was to develop a consistent method for transformation and construction of mutant H. parasuis. Natural transformation of H. parasuis was conducted using 10 different field isolates and an iscR (a HTH-type transcriptional regulator at the 3’ end of capsule gene cluster) deficient mutant was constructed in this study.
**Materials and methods**

**Bacterial strains and culture conditions**

The *H. parasuis* strains used in this study are listed in Table I. All of the strains were isolated from different farms in Zhejiang province, China. The serovar of the strains were determined by a newly developed multiplex polymerase chain reaction (PCR) that distinguished between all previously described serovars except 5 and 12 (16). The strains were cultured at 37°C in brain heart infusion (BHI) or on Tryptic Soy Agar (TSA) supplemented with 0.025% nicotinamide adenine dinucleotide (NAD) and 2% bovine serum.

**Plasmid construction**

Primers used in this study are listed in Table II, and a 9 bp USS fragment of *H. parasuis* is present in primers 1 and 6. To construct a suicide vector for transformation and generate iscR mutants, a 973 bp fragment of *H. parasuis* was amplified from genome DNA of the strains used in this study are listed in Table I. All of *H. parasuis* were grown in BHI and *Haemophilus parasuis* with little modification (17). When transformed with closed-circular or linearized plasmid DNA was done as described for *H. influenzae* with little modification (17). When transformed with closed-circular plasmid, 1 µg of circular recombinant plasmid pTR or pTRU was added to 1 mL of competent cells, mixed gently by inverting the tube 5 to 6 times, and then incubated at 37°C for 30 min. Then 80% glycerol was added to a final concentration of 30% to 32%, and incubated at 25°C for 10 min after inverting the tube 5 to 6 times. The bacteria were collected and resuspended in BHI before plating on TSA plus NAD and serum (containing 25 µg/mL kanamycin) immediately at proper dilution. The TSA plates were incubated at 37°C for 24 to 72 h. Transformation frequencies were calculated by CFU mL⁻¹ on TSA with kanamycin divided by CFU mL⁻¹ on TSA without kanamycin.

When transformed with linearized plasmid, 1 µg of *BamHI* linearized pTRU was mixed with 1 mL of competent cells, and the mixture was incubated at 37°C for 15 min. Cells were harvested and resuspended in BHI before plating on TSA plus NAD and serum (containing 25 µg/mL kanamycin) immediately at proper dilution. The TSA plates were incubated at 37°C for 24 to 72 h, and transformation frequencies were calculated as described above.

The 10 randomly selected strains were also subjected to electroporation and another natural transformation procedure with pTRU as described (4,8).

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**Media-IV(M-IV) medium induction of competence**

Competence was induced by transferring *H. parasuis* from BHI to M-IV medium (11). *Haemophilus parasuis* strains were retrieved from −80°C and inoculated in BHI supplemented with NAD and bovine serum at 1:100 dilution. The bacteria were cultured at 37°C by rotating at 200 rounds per minute (rpm) in a shaker incubator. When the optical density at 600 nm (OD₆₀₀) reaches 0.1−0.2, the bacteria were collected by centrifugation and washed once in equal volume of M-IV. The bacteria was resuspended in equal volume of M-IV and incubated at 37°C by rotating at 200 rpm for 100 min in a shaker incubator. Then cells were collected and resuspended in an equal volume of M-IV medium for the transformation assay.

**Transformation procedure**

Transformation with closed-circular or linearized plasmid DNA was done as described for *H. influenzae* with little modification (17). When transformed with closed-circular plasmid, 1 µg of circular recombinant plasmid pTR or pTRU was added to 1 mL of competent cells, mixed gently by inverting the tube 5 to 6 times, and then incubated at 37°C for 30 min. Then 80% glycerol was added to a final concentration of 30% to 32%, and incubated at 25°C for 10 min after inverting the tube 5 to 6 times. The bacteria were collected and resuspended in BHI before plating on TSA plus NAD and serum (containing 25 µg/mL kanamycin) immediately at proper dilution. The TSA plates were incubated at 37°C for 24 to 72 h. Transformation frequencies were calculated by CFU mL⁻¹ on TSA with kanamycin divided by CFU mL⁻¹ on TSA without kanamycin.

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The 10 randomly selected strains were also subjected to electroporation and another natural transformation procedure with pTRU as described (4,8).
Transformation assays

Three *H. parasuis* strains were selected for optimizing transformation conditions. To determine the optimum growth stage for development of competence, *H. parasuis* competence was induced at various growth stages measured by OD$_{600}$ and competent cells were transformed using pTRU. To determine the effect of USS sequence on transformation frequency, *H. parasuis* competence was induced when OD$_{600}$ reached 0.1–0.2, and competent cells were transformed using pTR or pTRU. Comparison of transformation efficiency using pTR or pTRU was analyzed by one-way ANOVA using computer software (SPSS Statistics, version 17.0; IBM, USA), data is considered statistically significant when $P \leq 0.05$.

To measure the occurrence of natural competence among *H. parasuis* strains, 10 strains were selected randomly, and competence was induced at OD$_{600}$ of 0.2, then transformed with circular or BamH I linearized pTRU.

Identification of iscR deficient mutants of *H. parasuis*

To check the reliability of homologous recombination in *H. parasuis*, 6 strains were transformed using circular or linearized pTRU, and genome DNA of 20 kanamycin resistant colonies were extracted by proteinase K digestion for each strain (18). Primers P3/P4, P11/P12 were used to check the insertion of kanamycin gene and deletion of iscR gene in genome DNA, and M13 primers were used to check the absence of pTRU in the bacteria. Replacement of iscR gene by the kanamycin gene in the chromosome was further confirmed by sequencing the PCR amplicon with primers P9 and P10, which are located at 1349 bp ahead of the upstream homologous arm and 278 bp behind the downstream homologous arm, respectively.

Results

Competence development at different growth stage

Competence of *H. parasuis* strains were induced at various cell concentrations (OD$_{600}$ 0.1 to 1.0) and competent cells were transformed using pTR. Transformation frequency was highest at OD$_{600}$ 0.1 to 0.2, and dropped continuously with the increase in cell concentration (Figure 2). Extensive loss of competence development ability was observed when OD$_{600}$ reached 0.8 to 1.0.

Effect of USS on natural transformation of *H. parasuis*

Two recombinant plasmid pTRU (containing 2 extra USS) and pTR were transformed into *H. parasuis* under the same conditions. Transformation efficiency of pTRU was slightly higher than that of pTR in ZJ0901 and ZJ1017, and lower in ZJ1307 (Figure 3). No significant difference was found between the 2 plasmids in transformation efficiency ($P > 0.05$). Analysis of the pTR gene using computer software (MegAlign, DNASTAR Lasergene; DNASTAR, Madison, Wisconsin, USA) revealed no homologous sequence of any of the 2 reported USS sequences (7,8) and its complement sequence. The presence of the USS in pTRU did not facilitate the transformation of *H. parasuis*.

Natural competence among *H. parasuis* strains

Ten randomly selected field strains were transformed with circular or linearized pTRU. It shows that 6 out of 10 strains could be transformed with both circular and linearized pTRU by using the M-IV procedure. Transformation frequency varies from $4.1 \times 10^{-5}$ to $1.1 \times 10^{-4}$ when using circular plasmid, and increases about 2- to 3-fold when transforming with linearized plasmid (Figure 4). Among the rest, 4 strains that could not be transformed with circular plasmid, 2 of them (ZJ1004 and ZJ1209) could be transformed using linearized pTRU at very low efficiency. Extensive difference in transformation efficiency was observed among strains (Figure 4).

Efficient targeted gene replacement of *H. parasuis* by natural transformation

The deletion of iscR and insertion of kanamycin gene were determined by PCR. It shows that the iscR gene was replaced by the kanamycin gene in the *H. parasuis* chromosome, and the iscR mutant shows genetic stability for at least 20 passages on TSA in the presence or absence of kanamycin (Figure 5). Twenty kanamycin resistant clones from each of the 6 strains transformed with circular
USs does not create a practical barrier to efficient transformation in *H. parasuis* has no effect on transformation efficiency of M-IV. USs of nucleotide sequence (7,8). But our result shows that the reported lytica, was identified in the SH0165 genome (21). The effect of USs on *Actinobacillus pleuropneumoniae*, identical to *H. parasuis* conserved (20). One USs signal sequence of, and is evolutionary Pasteurellaceae genomes of several members of *M-IV* induction of competence in *H. influenzae* to 0.2), which is similar to the conditions known to be optimal for *H. influenzae* *H.*. and developed a simple method for gen-

Figure 5. Identification of ΔiscR mutants. The PCR analysis of genomic DNA of the 2nd (P2) and 20th (P20) passages of ΔiscR mutants with wild type *H. parasuis* (HPs-WT) and pTRU as control. Lane 1—primers P3 and P4 were used to identify the insertion of kanamycin gene (816 bp) in chromosome. Lane 2—primers P11 and P12 were used to identify the deletion of iscR (447 bp) gene from chromosome. Lane 3—primers S1 and S2 were used to check the replacement of iscR gene with kanamycin gene, and the size of PCR product increases from 1265 bp to 1634 bp after the replacement. Lane 4—primers M13-F and M-13-R were used to check the suicide of pTRU after homologous recombination took place. Lane 5—HP1F3/HP2F2 and HPRevx are *H. parasuis* species specific primers with amplicon size of approximately 1090 bp (19). Lane M—250 bp DNA ladder.

or linearized pTRU were checked, and over 90% of the clones were iscR mutant. Interestingly, targeted gene replacement only took place in the 6 strains that could be transformed using both circular and linearized plasmid, but not in strains (ZJ1004 and ZJ1209), which could be only transformed using linearized plasmid. In these 2 strains (ZJ1004 and ZJ1209), incomplete homologous recombination may occur, and the kanamycin gene and iscR gene were both detected in the recombinants obtained from the linearized plasmid. In addition, the suicide of the pTRU in *H. parasuis* was confirmed by PCR with M13 primers (Figure 5).

### Discussion

Although increases in potential virulence factors have been found, pathogenesis of *H. parasuis* is poorly understood. Identification of these factors involved in pathogenesis has been limited due to a lack of general methods for producing mutants. In this study, we found the applicability of the defined M-IV medium in inducing competence of *H. parasuis*, and developed a simple method for generating mutants. The M-IV medium was first developed for inducing competence in *H. influenzae* (11). Competence development of *H. parasuis* was better at an early exponential growth stage (OD₆₀₀ 0.1 to 0.2), which is similar to the conditions known to be optimal for induction of competence in *H. influenzae* (17).

The DNA uptake signal sequence is highly over-represented in genomes of several members of *Pasteurellaceae*, and is evolutionary conserved (20). One USS signal sequence of *H. parasuis*, identical to that found in *Actinobacillus pleuropneumoniae* and *Mannheimia haemolytica*, was identified in the SH0165 genome (21). The effect of USS on natural transformation of *H. parasuis* has been confirmed previously, although *H. parasuis* strains may differ in requirements of the USS nucleotide sequence (7,8). But our result shows that the reported USS of *H. parasuis* has no effect on transformation efficiency of M-IV medium-induced competent cells. Similarly, it is believed that the USS does not create a practical barrier to efficient transformation in *Gallibacterium anatis* (*G. anatis*), and plasmids with no USS sequences could be well transformed into the bacteria (15).

It is believed that the competence development of *H. influenzae* is due to a starvation response. In *H. influenzae*, competence is induced with the starvation medium and absolutely requires an increase in cAMP, an established indicator of nutritional stress, but excessive cAMP may interfere with competence induction (22). In *H. parasuis*, the effect of cAMP on natural transformation is controversal. Increasing transformation efficiency was observed when the concentration of cAMP supplementation rising from 0.05 to 8 mM, but no significant difference in transformation efficiency was observed in another report under similar cAMP concentration and transformation procedure (7,8). The genetic difference of strains used in a previous report may be responsible for the sensitivity of cAMP in inducing competence, and it had been shown that an icc deletion mutant of *H. influenzae* was much more sensitive to exogenous cAMP (22). We also tested the effect of cAMP on transformation efficiency in M-IV medium induced competent cells, and supplementation of 1 mM cAMP in either competence induction or transformation stage did not increase the transformation frequency. This may be because sufficient cAMP has been produced when cultured in starvation medium, extra cAMP is not necessary. It has been demonstrated that *E. coli* cAMP levels peak during the transition to starvation (23–25).

The variation in transformability was reported in several species. Only 18 of 31 serotype b isolates of *H. influenzae* were transformable, while 29 of 34 strains were transformable when the tested isolates were dominated by non-serotype b strains (26,27). In *G. anatis*, all 9 tested strains were transformable with chromosomal DNA or linearized gene disruption constructs, but only 6 of 9 were transformable using circular plasmid (15). Ten randomly selected *H. parasuis* strains were subjected to natural transformation, and 6 of them were found transformable using both circular and linearized plasmid. Moreover, 2 of the remaining 4 strains could be transformed using linearized plasmid. The increase of transformation frequencies was observed when using linearized instead of the circular pTRU, and the increase of transformants generated by linear plasmid was also observed when using a different natural transformation procedure (9). Only 1 of the 10 strains (ZJ1208) were transformable when subjected to the previously reported transformation procedure, which similarly reported that 1 of 11 tested strains were transformable (8). Electroporation was used to transform *H. parasuis*, but no kanamycin resistant clone was observed under our experiment conditions.

In this study, 2 serovar 7 strains were transformable using both circular and linearized plasmid; all 3 serovar 4 strains were transformable using linearized plasmid, but only 2 of them were transformable using circular plasmid. Only 1 of the 3 serovar 13 strains could be transformed using both forms of plasmid, while the other 2 strains were non-transformable with both forms of the plasmid. Hence, natural competence of these strains shows no strict association with serovar, but it is impossible to carry on a statistical analysis due to the limited number of strains tested in this study. Analysis with more strains in each serovar is needed to determine the relationship between natural competence and serovar.

Study of *H. parasuis* virulence factors has long been hindered by problems in generating mutants. The homologous recombination
was efficient in M-IV induced competent *H. parasuis*, and the iscR mutants were genetically stable. Moreover, another wza (capsular export protein) mutant was also generated by the procedure established in this study in our laboratory (28). Hence, the method established here allows the transformation of a larger spectrum of strains with an easily accessed plasmid, which will facilitate the identification of virulence factors and the characterization of pathogenicity of *H. parasuis*.

**Acknowledgment**

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**References**

Minimum dose, antigen content, and immunization duration of a trivalent vaccine of inactivated *Haemophilus parasuis* serovars 4, 5, and 12 against Glässer’s disease in pigs

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Abstract

The objective of this study was to assess the minimum dose, antigen content, and immunization duration of a trivalent vaccine containing inactivated *Haemophilus parasuis* serovars 4, 5, and 12 and the Montanide GEL 01 PR adjuvant in piglets and pregnant sows. Our results demonstrated that the minimum vaccine dose was 2 mL per pig and the optimal antigen content 2.0 × 10^9, 1.0 × 10^9, and 1.0 × 10^9 colony-forming units/mL of serovars 4, 5, and 12, respectively. The vaccine provided effective protection 14 d after the 2nd vaccination, and the period of immune protection was 180 d (6 mo) after the 2nd vaccination. Maternal antibodies provided early protection for the piglets, and vaccinating the sows before farrowing helped to control disease and protected the piglets during lactation; the piglets were protected during the finishing period by being vaccinated during lactation. Our findings provide a basis for developing a commercial trivalent vaccine of inactivated *H. parasuis* serovars 4, 5, and 12 against Glässer’s disease.

Résumé

L’objectif de la présente étude était d’évaluer la dose minimale, le contenu en antigène, et la durée d’immunisation d’un vaccin trivalent contenant les sérovars 4, 5, et 12 d’*Haemophilus parasuis* et l’adjuvant Montanide GEL 01 PR chez des porcelets et des truies gestantes. Nos résultats ont démontré que la dose minimale de vaccin était de 2 mL par porc et le contenu optimal en antigène était de 2.0 × 10^9, 1.0 × 10^9, and 1.0 × 10^9 unités formatrices de colonie/mL pour les sérovars 4, 5, et 12, respectivement. Le vaccin a fourni une protection efficace 14 j suite à la deuxième vaccination, et la période de protection immunitaire était de 180 j (6 mois) après la deuxième vaccination. Les anticorps maternels ont fourni une protection précoce aux porcelets, et la vaccination des truies avant la mise-bas aidait à maîtriser la maladie et a protégé les porcelets durant la lactation; les porcelets étaient protégés durant la période de finition en étant vaccinés durant la lactation. Nos trouvailles fournissent des éléments de base pour développer un vaccin trivalent commercial contre la maladie de Glässer avec des souches inactivées d’*H. parasuis* sérovars 4, 5, et 12.

Introduction

*Haemophilus parasuis*, which belongs to the *Pasteurellaceae* family, can cause severe infections of the upper respiratory tract, polyarthritis, meningitis, and arthritis (Glässer’s disease) in pigs (1). To date, *H. parasuis* has mainly caused significant losses among piglets 4 to 12 wk old (2,3). Fifteen serovars of *H. parasuis*, which range from highly virulent to nonvirulent, have been described by immunodiffusion tests and multiplex polymerase chain reaction (PCR). Sequence variations within the capsule loci can distinguish all 15 serovars except serovars 5 and 12 (1,4). Moreover, the pathogenicity and prevalence of different *H. parasuis* serovars may vary among regions and over time within a region (5–8).

Vaccination is generally considered to be the most effective means of controlling Glässer’s disease, which normally has high mortality and morbidity rates. Inactivated *H. parasuis* bacterin, which is used worldwide, can elicit efficient protection against challenge with a homologous serovar. However, disease control is limited because of the lack of effective vaccines against a broad spectrum of strains (9–11). Recently, subunit vaccines that comprise newly identified protective antigens, such as recombinant transferrin-binding protein B (TbpB), outer membrane protein (OMP) formulations enriched with TbpB, 4 MPs (OMP2, D15, PalA, and HPS-6257), or transferrin-binding protein A, have been proven to provide partial protection against *H. parasuis* challenge (9,10,12). All types of vaccines usually provide protection against challenge with homologous serovars, but few studies have reported cross-protection (2,12,13).

To date, vaccines against Glässer’s disease containing inactivated *H. parasuis* serovars 4 and 5, serovar 5, and serovars 1 and 6 are the primary commercial vaccines in China, the United States, and Spain.
respectively. These vaccines play an important role in preventing and controlling Glässer’s disease (14).

In a previous study we screened the strongly immunogenic serovars 4, 5, and 12 as candidate vaccine strains and compared the effectiveness of vaccines containing inactivated \( H. \text{parasuis} \) serovars 4, 5, and 12 when administered with a variety of adjuvants [traditional mineral oil, aluminum hydroxide, Montanide GEL 01 PR (a new adjuvant based on the dispersion of a high-molecular-weight polyaacrylic polymer in water; SEPPIC, Shanghai, China), Montanide IMS 1313N VG, and Montanide ISA 760 VG]. We concluded that the Montanide GEL 01 PR should be used as a candidate adjuvant, deserving further study because a vaccine administered with this adjuvant was safe, generated high concentrations of antibodies, and exhibited 100% protection (15). The objectives of the present study were to assess the minimum dose, antigen content, and immunization duration of a trivalent vaccine containing inactivated \( H. \text{parasuis} \) serovars 4, 5, and 12 administered to pigs for the first time and to provide a basis for developing a commercial trivalent vaccine with these inactivated serovars to protect against Glässer’s disease.

### Materials and methods

#### Animals

Female Landrace × Large White piglets aged 3 to 4 wk old and Landrace × Large White sows that were 8 to 9 wk pregnant were purchased from ShengPing Co., Ltd, Luoyang, China. A single nasal swab obtained from each pig was shown by PCR to be negative for \( H. \text{parasuis} \) as described previously (15). The pigs were determined to be serologically negative by a microagglutination test (MAT) developed in our laboratory. Briefly, 50 \( \mu \text{L} \) of sterile PBS was added to 96-well plates, along with 50 \( \mu \text{L} \) of test serum diluted 2-fold with sterile PBS. Into each well was mixed 50 \( \mu \text{L} \) of inactivated \( H. \text{parasuis} \) whole-cell antigen. The mixture was incubated at 37°C for 7 h and then at 4°C for 12 to 24 h. Titers ≥ 1:8 and ≤ 1:4 were considered to be positive and negative, respectively.

All the pigs were housed in isolation in pens with a concrete floor. Feed and water were provided \textit{ad libitum} throughout the study. All the animal procedures were conducted in compliance with the guidelines of the Animal Care and Use Committee of Henan University of Science and Technology, Luoyang, China.

#### Isolates of \( H. \text{parasuis} \)

Isolates of \( H. \text{parasuis} \), serovars 4, 5, and 12, that were screened by our laboratory (16) were used for vaccine production. The bacteria were cultured on tryptic soy agar (TSA) supplemented with nicotinamide adenine dinucleotide (NAD), 10 \( \mu \text{g/mL} \), and 5% fetal calf serum and incubated at 37°C for 48 h. The organisms were then washed off the TSA plates with sterile 15% skimmed milk powder and stored at -80°C until used.

#### Vaccine formulations and vaccination schedule

Four vaccines (F1 to F4) were compared. Briefly, \( H. \text{parasuis} \) serovars 4, 5, and 12 were enumerated by plate counts. Then the bacterial cultures were inactivated by incubation with 0.3% formaldehyde solution at 37°C for 48 h and pelleted by high-speed centrifugation. The pellets were resuspended in sterile phosphate-buffered saline (PBS) as whole-cell antigens and subsequently formulated with Montanide GEL 01 PR adjuvant (kindly provided by SEPPIC) at an 85:15 ratio (\( H. \text{parasuis} \)adjuvant) in accordance with the manufacturer’s instructions. The vaccines contained serovars 4, 5, and 12 at the following antigen concentrations: F1, \( 2.0 \times 10^8 \), \( 1.0 \times 10^9 \), and \( 1.0 \times 10^9 \) colony-forming units (CFU)/mL, respectively; F2, \( 1.0 \times 10^8 \), \( 5.0 \times 10^9 \), and \( 5.0 \times 10^9 \) CFU/mL, respectively; F3, \( 5.0 \times 10^8 \), \( 2.5 \times 10^9 \), and \( 2.5 \times 10^9 \) CFU/mL, respectively; and F4, \( 2.5 \times 10^8 \), \( 1.3 \times 10^9 \), and \( 1.3 \times 10^9 \) CFU/mL, respectively. The physical properties and sterility of the vaccines were described by the Veterinary Biological Products Procedures of the People’s Republic of China.

All the vaccines were administered intramuscularly twice (21 d apart) in the neck. The day of the 1st vaccination was designated as d 0. At 14 d after the 2nd vaccination all the piglets were challenged intraperitoneally with \( 9.0 \times 10^9 \) CFU (15) of \( H. \text{parasuis} \) serovar 4 (approximately 3 mL), \( 5.2 \times 10^8 \) CFU (15) of \( H. \text{parasuis} \) serovar 5 (approximately 2 mL), and \( 3.5 \times 10^8 \) CFU (15) of \( H. \text{parasuis} \) serovar 12 (approximately 1.5 mL); the bacteria had been cultured in trypticase soy broth supplemented with NAD, 10 \( \mu \text{g/mL} \), and 5% fetal calf serum for 16 h. Control pigs were injected with 2 mL of sterile PBS on the same day that the other groups were vaccinated. Blood samples from the piglets were collected by puncture of the jugular vein.

#### Minimum vaccine dose for piglets

Sixty piglets were randomly assigned to 12 groups (A1 to A12) of 5 animals each. The piglets in groups A1 to A3, A4 to A6, and A7 to A9 were vaccinated with 2, 1, and 0.5 mL, respectively, of the F1 vaccine. The piglets in groups A10 to A12 served as controls. Groups A1, A4, A7, and A10 were challenged with \( H. \text{parasuis} \) serovar 4; groups A2, A5, A8, and A11 were challenged with serovar 5; and groups A3, A6, A9, and A12 were challenged with serovar 12.

Blood samples from the piglets were collected before challenge (on d 35). The serum was stored at ~80°C until the MAT analysis. The experiment was terminated 15 d after challenge; survivors were killed by intravenous administration of sodium pentobarbital. Piglets found moribund during the experiment were humanely killed in the same manner. All of the animals underwent necropsy; gross lesions were recorded (special attention was paid to the pleural, pericardial, and peritoneal cavities, the lungs, and the central nervous system), and samples were obtained for bacterial isolation.

#### Minimum vaccine dose for pregnant sows

Twenty pregnant sows were randomly assigned to 4 groups (B1 to B4) of 5 animals each. The sows in groups B1, B2, and B3 were vaccinated with 2, 1, and 0.5 mL, respectively, of the F1 vaccine. The sows in groups B4 served as controls.

Blood samples from piglets born to the pregnant sows were collected at 1, 4, 7, 10, 15, 20, 25, 30, 35, 40, 45, and 50 d of age. Serum antibody titers were analyzed to assess dynamic changes in the \( H. \text{parasuis} \) antibody levels in these piglets. Fifteen piglets 21 to 22 d old were selected from each group of sows. Then 15 piglets were randomly assigned to 3 groups of 5 piglets each, and immediately challenged with \( H. \text{parasuis} \) serovars 4, 5, and 12, respectively. The procedures after challenge were as described.
Effect of vaccine antigen content on piglets

Seventy-five piglets were randomly assigned to 15 groups (C1 to C15) of 5 animals each. The piglets in groups C1 to C3, C4 to C6, C7 to C9, and C10 to C12 were vaccinated with 2 mL of the F1, F2, F3, and F4 vaccines, respectively. The piglets in groups C13 to C15 served as controls. Groups C1, C4, C7, C10, and C13 were challenged with serovar 4; groups C2, C5, C8, C11, and C14 were challenged with serovar 5; and groups C3, C6, C9, C12, and C15 were challenged with serovar 12. Blood samples from the piglets were collected before challenge (on d 35), and the serum was stored at −80°C until the MAT analysis. The procedures after challenge were as described.

Effect of vaccine antigen content on pregnant sows

Twenty-five pregnant sows were randomly assigned to 4 groups (D1 to D5) of 5 animals each. The sows in groups D1, D2, D3, and D4 were vaccinated with 2 mL of the F1, F2, F3, and F4 vaccines, respectively. The sows in group D5 served as controls. The procedures on the piglets born to these sows were as described.

Immunization duration in piglets

One hundred and fifty piglets were randomly assigned to 2 groups (E1 and E2) of 75 animals each. The piglets in group E1 were vaccinated with 2 mL of the F1 vaccine. The piglets in group E2 served as controls. Blood samples were collected on days 0 and 21 after the 1st vaccination and on days 7, 14, 28, 60, 90, 120, 150, 180, 210, and 240 after the 2nd vaccination. Serum titers of H. parasuis antibodies were analyzed to assess dynamic changes in the levels.

Fifteen piglets were selected from each group at 14, 90, 180, and 210 d after the 2nd vaccination and then randomly assigned to 3 groups of 5 piglets each. The piglets were immediately challenged with H. parasuis serovars 4, 5, and 12, respectively. The procedures after challenge were as described.

Immunization duration in pregnant sows

Twelve pregnant sows were randomly assigned to 2 groups (Z1 and Z2) of 6 animals each. The sows in group Z1 were vaccinated with 2 mL of the F1 vaccine. Group Z2 served as controls. Blood samples from the sows were collected on days 0 and 21 after the 1st vaccination and on days 7, 14, 28, 60, 90, 120, 150, 180, 210, and 240 after the 2nd vaccination. Serum titers of H. parasuis antibody were analyzed to assess dynamic changes in the levels. Fifteen piglets aged 21 to 22 d, 34 to 36 d, and 56 to 58 d were selected from each group and then randomly assigned to 3 groups of 5 piglets each and immediately challenged with H. parasuis serovars 4, 5, and 12, respectively. The procedures after challenge were as described.

Statistical analysis

Statistical analysis was done by means of 1-way analysis of variance. A P-value < 0.05 was considered to be significant. The analysis was conducted with the use of SPSS Statistics for Windows, version 17.0 (SPSS, Chicago, Illinois, USA).

Results

As Table I shows for the vaccine-dose experiments, among the piglets the mean titers of antibody to serovars 4, 5, and 12 were significantly higher (P < 0.05) in groups A1 to A3 (vaccinated with 2 mL of the F1 vaccine) than in groups A4 to A6 and groups A7 to A9 (vaccinated with 1 mL and 0.5 mL of the F1 vaccine, respectively). The mean titers did not differ significantly between groups A4 to A6 and groups A7 to A9. No H. parasuis antibodies were observed in groups A10 to A12, the unvaccinated control piglets. After challenge with H. parasuis serovars 4, 5, and 12, all the control piglets had

Table I. Protective efficacy of the F1 vaccinea against serovars 4, 5, and 12 of Haemophilus parasuis and mean titers of antibody before challenge in piglets that had received various doses of the vaccine

<table>
<thead>
<tr>
<th>Piglet group</th>
<th>Number of piglets</th>
<th>Vaccine dose (mL)</th>
<th>Protective efficacy (and mean antibody titerb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7 to A9</td>
<td>15</td>
<td>0.5</td>
<td>Serovar 4: 60% (1:12) Serovar 5: 60% (1:11) Serovar 12: 60% (1:12)</td>
</tr>
<tr>
<td>A4 to A6</td>
<td>15</td>
<td>1</td>
<td>100% (1:18) 80% (1:16) 100% (1:18)</td>
</tr>
<tr>
<td>A1 to A3</td>
<td>15</td>
<td>2</td>
<td>100% (1:28) 100% (1:37) 100% (1:37)</td>
</tr>
<tr>
<td>Controlsc (A10 to A12)</td>
<td>15</td>
<td>2</td>
<td>0% (—) 0% (—) 0% (—)</td>
</tr>
</tbody>
</table>

a The vaccine, given as 2 intramuscular doses 21 d apart, contained serovars 4, 5, and 12 of H. parasuis at antigen contents of 2.0 × 10⁹, 1.0 × 10⁹, and 1.0 × 10⁹ colony-forming units (CFU)/mL, respectively. The piglets were challenged intraperitoneally with H. parasuis 14 d after the second vaccination.

b The mean antibody titers in groups A1 to A3 were significantly higher (P < 0.05) than those in the other experimental groups.

c The piglets in the control group were injected with 2 mL of sterile phosphate-buffered saline the same days as the other groups were vaccinated. The dash indicates that no H. parasuis antibodies were detected.
Table II. Mean titers of maternal antibody to *H. parasuis* serovars 4, 5, and 12 in piglets born to pregnant sows that had received various doses of the F1 vaccine

<table>
<thead>
<tr>
<th>Piglet age (days)</th>
<th>Vaccine dose, mL (and sow group); mean antibody titer a</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serovar 4</td>
<td>0.5 (B3)</td>
</tr>
<tr>
<td>1</td>
<td>1:4</td>
<td>1:4</td>
</tr>
<tr>
<td>4</td>
<td>1:4</td>
<td>1:4</td>
</tr>
<tr>
<td>7</td>
<td>1:8</td>
<td>1:8</td>
</tr>
<tr>
<td>10</td>
<td>1:16</td>
<td>1:16</td>
</tr>
<tr>
<td>15</td>
<td>1:32</td>
<td>1:32</td>
</tr>
<tr>
<td>20</td>
<td>1:32</td>
<td>1:32</td>
</tr>
<tr>
<td>25</td>
<td>1:32</td>
<td>1:32</td>
</tr>
<tr>
<td>30</td>
<td>1:16</td>
<td>1:16</td>
</tr>
<tr>
<td>35</td>
<td>1:16</td>
<td>1:16</td>
</tr>
<tr>
<td>40</td>
<td>1:8</td>
<td>1:8</td>
</tr>
<tr>
<td>45</td>
<td>1:4</td>
<td>1:2</td>
</tr>
<tr>
<td>50</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a Antibody titers ≥ 1:8 and ≤ 1:4 were deemed to be positive and negative, respectively. The mean titer in the piglets born to the B1 group was not significantly different from that of the piglets born to the B2 group but was significantly higher (P < 0.05) than that of the piglets born to the B3 group. The mean titers in the piglets born to the B2 and B3 groups did not differ significantly. The dash indicates that no *H. parasuis* antibodies were detected.

In the antigen-content experiments the mean titers of antibody to serovars 4, 5, and 12 in the piglets (Table III) were significantly higher (P < 0.05) in groups C1 to C3 (vaccinated with the F1 vaccine) than in groups C4 to C6 (vaccinated with the F2 vaccine). The antibody titers in those groups were significantly higher (P < 0.05) than the titers in groups C7 to C9 and C10 to C12 (vaccinated with the F3 and F4 vaccines, respectively). The mean titers did not differ significantly between groups C7 to C9 and groups C10 to C12. No antibodies against *H. parasuis* serovars 4, 5, and 12 were observed in the control piglets (groups C13 to C15), all of which showed signs of Glässer’s disease after challenge and died by 72 h after challenge. The F1 vaccine provided 100% protection against challenge with *H. parasuis* serovars 4, 5, and 12, and the F2 vaccine provided 100% protection against challenge with *H. parasuis* serovars 5 and 12 but only 80% protection against serovar 4, whereas the F3 and F4 vaccines provided little protection against the 3 serovars. The organism was isolated from tissues and organs of all of the dead experimental piglets, which exhibited symptoms and pathological changes of Glässer’s disease in the upper respiratory tract similar to those in the controls. None of the surviving piglets exhibited similar symptoms or pathological changes.

Dynamic changes in the antibody titers of the piglets born to the pregnant sows are shown in Table II for the vaccine-dose experiments. The mean titers in the piglets born to the B1 and B2 groups (vaccinated with 2 mL and 1 mL of the F1 vaccine, respectively) were highest at day 20 and gradually decreased thereafter. However, the titers decreased faster in the B2 group than in the B1 group. The mean titer in the piglets born to the B3 group (vaccinated with 0.5 mL of the F1 vaccine) was highest at day 15 and then gradually decreased; however, the titers in those groups were the lowest (≤ 1:16) and were maintained for a short time. The mean titer in the piglets born to the B1 group was not significantly different from that of the piglets born to the B2 group but was significantly higher (P < 0.05) than that of the piglets born to the B3 group. The mean titer in the piglets born to the B2 group was not significantly different from that of the B3 group. No maternal antibodies were observed in the controls (group B4). Among the 15 piglets 21 to 22 d old that were selected from each group and challenged with *H. parasuis* serovars 4, 5, and 12 the protective efficacy of the vaccine was the same as for groups A1 to A3, A4 to A6, A7 to A9, and A10 to A12.
Dynamic changes in the titers of antibody to 

H. parasuis

serovars 4, 5, and 12 in the piglets in the immunization-duration experiment are shown in Table IV. The mean titers gradually increased from the 1st vaccination to day 28 after the 2nd vaccination and then gradually decreased, disappearing by day 210. The piglets were challenged with 

H. parasuis

serovars 4, 5, and 12 at 14, 90, 180, and 210 d after the 2nd vaccination. One piglet challenged with 

H. parasuis

serovar 4 died 10 d after challenge at d 210, whereas all of the other experimental piglets survived; all of the control piglets had signs of Glässer’s disease and died by 72 h after challenge. The organism was isolated from tissues and organs of the dead experimental piglets, which exhibited symptoms and pathological changes of Glässer’s disease in the upper respiratory tract similar to those in the control groups. None of the surviving piglets exhibited similar symptoms and pathological changes.

Dynamic changes in the titers of antibody to 

H. parasuis

serovar 4, 5, and 12 in the pregnant sows are shown in Table V. The titers increased more slowly than those in the piglets represented in Table IV from the 1st vaccination to day 14 after the 2nd vaccination, but other changes were similar to those for the piglets. Dynamic changes in the titers of maternal antibody in the piglets born to pregnant sows were identical to those in the B1 group (vaccinated with 2 mL of the F1 vaccine) as shown in Table II. All the piglets aged 21 to 22 d and 60% of those aged 34 to 36 d survived challenge with 

H. parasuis

serovars 4, 5, and 12, whereas all of the piglets aged 56 to 58 d, as well as the control piglets, died after challenge. The organism was isolated from tissues and organs of all of the dead piglets, which had signs of Glässer’s disease. None of the surviving piglets exhibited symptoms and pathological changes of Glässer’s disease in the upper respiratory tract, as occurred in the control group.
that the optimal antigen contents of H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when the concentration of antibodies against H. parasuis serovars 4, 5, and 12 increased with increasing antigen content: the antibody titers induced by 2 mL of the F1 vaccine were significantly higher when

The minimum vaccine dose and optimal antigen content of the trivalent vaccine containing inactivated H. parasuis serovars 4, 5, and 12 were used to assess the immunization duration in piglets and pregnant sows. In these experiments, antibody titers of 1:16 or greater were observed from 14 to 180 d after the 2nd vaccination, and all of the piglets survived challenge with H. parasuis serovars 4, 5, and 12 at 14, 90, and 180 d after the 2nd vaccination. These results indicate that the trivalent vaccine provided effective protection at d 14 after the 2nd vaccination and that the immune protection lasted until 180 d (6 mo) after the 2nd vaccination. Our results also indicate that the 1st vaccination should be done in sows that have been pregnant for 8 to 9 wk, because high antibody concentrations could be detected in the vaccinated pregnant sows during farrowing. Additionally, sows should be given a booster injection 21 d later, because neonatal piglets could receive more maternal antibodies that allow them to survive without resulting disease while they produce an active immune response.

The maternal antibodies that are induced by commercially available bacterin containing H. parasuis serovars 2, 3, and 5 can provide early protection for piglets (18–20). The results obtained with the piglets born to vaccinated sows in this study support this view. Additionally, we detected maternal antibodies in neonatal piglets and dynamic changes in the antibody titers in vaccinated piglets aged 3 to 9 wk, as well as in pregnant sows. Our findings suggest that the maternal antibody titers were maintained for 6 wk (Table II) and therefore that vaccinating sows before farrowing may help to control the disease and protect the piglets during lactation. However, the susceptibility to H. parasuis infection and disease increased in piglets after weaning because of the disappearance of maternal antibodies and the loss of passive protection. Therefore, it is important that piglets be inoculated with an effective vaccine before the disappearance of maternal antibodies. In this study the maternal antibody concentration was highest in the piglets at 20 d of age and then gradually decreased. Thus, we recommend that the 1st vaccination be done in piglets 3 to 4 wk old and that they be given a booster injection 21 d later, as

### Table IV. Dynamic changes in the mean titers of antibody to H. parasuis serovars 4, 5, and 12 in 3 groups of 5 piglets each that received 2 mL of the F1 vaccine (E1) and 15 controls (E2)

<table>
<thead>
<tr>
<th>Serovar group</th>
<th>Day after first vaccination (0 or 21)</th>
<th>Mean antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>0  21  7  14  28  60  90  120  150  180  210  240</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>0  21  7  14  28  60  90  120  150  180  210  240</td>
<td></td>
</tr>
</tbody>
</table>

**Table V. Dynamic changes in the mean titers of antibody to H. parasuis serovars 4, 5, and 12 in pregnant sows, 6 of which received 2 mL of the F1 vaccine (Z1) while the other 6 served as controls (Z2)**

<table>
<thead>
<tr>
<th>Serovar group</th>
<th>Day after first vaccination (0 or 21)</th>
<th>Mean antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z1</td>
<td>0  21  7  14  28  60  90  120  150  180  210  240</td>
<td></td>
</tr>
<tr>
<td>Z2</td>
<td>0  21  7  14  28  60  90  120  150  180  210  240</td>
<td></td>
</tr>
</tbody>
</table>

**Note:**
- The vaccine was given as 2 intramuscular doses 21 d apart.
- The controls were injected with 2 mL of sterile phosphate-buffered saline the same days as the other piglets were vaccinated.
- Antibody titers ≥ 1:8 and ≤ 1:4 were deemed to be positive and negative, respectively. The dash indicates that no H. parasuis antibodies were detected.
this vaccination regimen provided protection against *H. parasuis* serovars 4, 5, and 12 for 180 d. Our results showed that the vaccinated offspring of the vaccinated sows had longer-lasting serologic protection, which would help to prevent *H. parasuis* colonization.

Our findings provide new, detailed data regarding the vaccine optimization that is needed to guarantee the efficacy of a trivalent vaccine against Glässer’s disease in piglets and pregnant sows containing inactivated *H. parasuis* serovars 4, 5, and 12, and they provide a basis for developing a commercial vaccine. Meanwhile, the practical significance of our findings is that if *H. parasuis* infections are endemic in a swine-breeding facility it is necessary to vaccinate the sows before farrowing. The piglets could be protected during the finishing period if they are vaccinated during lactation. Field trials of the trivalent vaccine that we developed, administered with Montanide GEL 01 PR adjuvant, should be conducted on pig farms in the future.

**Conflict of interest statement**

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

**Acknowledgments**

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**References**

Evaluation of adipose-derived stromal vascular fraction from the lateral tailhead, inguinal region, and mesentery of horses

Garrett L. Metcalf, Scott R. McClure, Jesse M. Hostetter, Rudy F. Martinez, Chong Wang

Abstract

Use of mesenchymal stem cells (MSCs) found in the stromal vascular fraction (SVF) of equine adipose tissue has promising applications for regenerative therapies. The most commonly used source of equine adipose tissue is the subcutaneous tailhead. The objective of this study was to compare 3 adipose depot sites in horses and determine the viability and cellular yield, capillary density, gene expression for selected markers, and colony-forming unit fibroblasts (CFU-Fs) in adipose tissue taken from these sites. Adipose tissue was excised from the area lateral to the tailhead, the inguinal region, and the small colon mesentery of 6 horses. Lipoaspirate was also collected from the area lateral to the tailhead. Stromal vascular fraction (SVF) was prepared in duplicate from the 3 different adipose tissue depots. The total nucleated and dead cell counts were determined manually using a hemocytometer and percent viability was calculated. Mass and volume of adipose were determined in order to calculate density and factor-VIII immunohistochemical staining was used to determine vascular density in the excisional adipose tissue samples from each horse. Quantitative polymerase chain reaction (qPCR) was used to quantify gene expression for selected cellular markers from each site. There were significant differences in viability, yield of nucleated cells/gram of adipose tissue, vascular density, gene expression, and CFU-Fs among adipose depots. Adipose from the mesentery yielded the highest number of nucleated cells/gram of tissue and the highest vascular density and percentage of CFU-Fs. In the horse, both the anatomical site of collection and the method of tissue collection significantly impact the yield and composition of cells in the SVF. Further study is needed to assess whether one adipose source is superior for harvesting mesenchymal stem cells (MSCs) and whether the differences among sources are clinically relevant for in-vivo treatment of musculoskeletal injuries in horses.

Résumé

L’utilisation de cellules souches mésenchymateuses (CSMs) retrouvées dans la fraction du stroma vasculaire (FSV) du tissu adipeux équin a des applications prometteuses pour les thérapies régénératrices. La source la plus fréquemment utilisée de tissu adipeux équin est le tissu sous-cutané de la base de la queue. L’objectif de la présente étude était de comparer trois sites de dépôts adipeux chez le cheval et de déterminer la viabilité et la récolte cellulaire, la densité capillaire, l’expression génique de marqueurs sélectionnés, et le nombre de fibroblastes formateur des colonies (FFC) dans le tissu adipeux prélevés de ces sites. Le tissu adipeux a été excisé de la région latérale à la base de la queue, de la région inguinale, et du mésentère du petit colon de six chevaux. Des aspirations de lipide ont également été prélevées de la région latérale de la base de la queue. La FSV a été préparée en duplicata à partir de chacun des trois dépôts différents de tissu adipeux. Les dénombrements totaux des cellules nucléées et mortes ont été déterminés manuellement à l’aide d’un hémocytomètre et le pourcentage de viabilité calculé. La masse et le volume de tissu adipeux ont été déterminés afin de calculer la densité et la coloration par immunohistochimie du facteur VIII a été utilisée afin de déterminer la densité vasculaire dans les échantillons de tissu adipeux excisé de chaque cheval. Une réaction d’amplification en chaîne par la polymérase quantitatives (ACPq) a été utilisée pour quantifier l’expression génique pour des marqueurs cellulaires sélectionnés de chaque site. Il y avait des différences significatives dans la viabilité, le rendement de cellules nucléées/gramme de tissu adipeux, la densité vasculaire, l’expression génique, et les FFCs entre les dépôts adipeux. Le tissu adipeux provenant du mésentère a généré le plus grand nombre de cellules nucléées grammes de tissu et la plus haute densité vasculaire et pourcentage de FFCs. Chez le cheval, le site anatomique de prélèvement et la méthode de prélèvement du tissu ont un impact significatif sur le rendement et la composition cellulaire dans la FSV. Des études additionnelles sont requises pour évaluer si une source de tissu adipeux est supérieure pour récolter des cellules souches mésenchymateuses et si les différences entre les sources sont cliniquement pertinentes pour le traitement in vivo de blessures morpho-squelettiques chez les chevaux.

(Traduit par Docteur Serge Messier)
Regenerative cell-based therapy is a promising tool for treating musculoskeletal injuries in equine athletes and has the potential to regenerate or modulate healing of tissue. Superficial digital flexor tenosynovitis is an example of a common injury in the horse industry with poor healing, long convalescence periods, loss of earnings, and high re-injury rates either in the same or contralateral limb (1–3). Mesenchymal stem cells (MSCs) have potential for treating musculoskeletal injuries in horses in a wide variety of disciplines (4–7).

There are numerous tissues that contain MSCs, but the most practical and widely used tissues are bone marrow and adipose. There are also many techniques for obtaining MSCs. The most commonly used techniques are culture-expanded bone marrow and adipose-derived stem cells in the stromal vascular fraction (SVF), which contains mesenchymal stem cells and a variety of nucleated cells that can contribute to tissue healing (8–10). Since there are relatively few MSCs in bone marrow, culture expansion is indicated to obtain the desired number of MSCs for implantation (8,10,11). There are data that support MSCs derived from bone marrow for specific applications (12). When the SVF is used for immediate implantation, there is potential for high MSC yield.

Data indicate that up to 222 times more MSCs are found in adipose tissue than in equal amounts of bone marrow in the horse and in humans up to 500 times more MSCs are found in adipose than in bone marrow (8). Because adipose tissue has a relatively high density of MSCs, fresh SVF can be used for immediate implantation without expansion, which is an attractive feature of SVF regenerative therapy (13,14). A number of factors must be considered when choosing the source and technique, including timing of implantation and time required to obtain cells, harvest location, patient morbidity, and efficacy. These factors affect the clinician’s selection of cultured MSCs or fresh SVF. Regardless of the source, the objective is to maximize the MSC harvest.

When bone marrow is used, it has been shown that different harvest locations vary in nucleated cell counts (15). It is not known, however, if this is also true for adipose harvest sites. Adipose is an abundant source of mesenchymal stem cells, with depots in the horse found lateral to the tailhead, the inguinal region, neck, omentum, and mesentery. Historically, the harvest site of choice for adipose-derived mesenchymal stem cells (MSCs) or stromal vascular fraction (SVF) in horses is lateral to the tailhead (16–18). Fat lateral to the tailhead varies in vascular density, which has in turn been shown to correlate with MSC colony-forming units (CFUs) in vitro (16). There is limited information, however, on the optimal location for harvesting adipose to obtain the SVF in horses and other species.

Certain clinical situations can dictate the harvest site of adipose tissues, including cosmetic appearance, convenience, adequate adipose depot for collection, and positioning of a horse that is already under general anesthesia. The concern with using alternative sites for adipose harvesting is the quality and quantity of cell yield. It has been found in humans that not all fat depot sites exhibit equivalent characteristics and there are differences in surface marker expression, doubling time, and SVF yield among subcutaneous, omental, and intrathoracic fat depot sites (19). Additionally, use of lipoaspiration can speed the adipose recovery process and result in a very small incision lateral to the tailhead, which improves cosmetic outcome and decreases potential morbidity, although the lipoaspiration procedure itself could affect cell yield and viability (18).

The objective of this study was to evaluate the nucleated cell fractions in adipose tissue obtained from the tailhead (excisional and lipoaspirate), inguinal region, and mesentery of the same horse and compare the viability and cellular yield, tissue and vascular density, gene expression for selected markers, and CFU-F in this tissue. We hypothesized that adipose depot sites with higher vascular density would yield a higher concentration of nucleated cells than sites with lower vascular density.

### Materials and methods

#### Animals

Six healthy horses were used in this study, as determined by physical examination and known health histories. It was required that the horses not have any prior history or evidence of intra-abdominal surgery or asymmetry over the gluteal or tailhead region. The median age was 3 y (1 to 15 y) and mean ± standard deviation (SD) weight was 482 ± 86.1 kg (318 to 559 kg). Breed distribution was 5 Quarter Horses and 1 Missouri Fox Trotter. All horses had been euthanized for reasons not associated with this study. Euthanasia procedures were approved by the Iowa State University Institutional Animal Care and Use Committee, which complies with the American Veterinary and Medical Association (AVMA) Guidelines for the Euthanasia of Animals.

#### Collection of adipose tissue

Following euthanasia, sites over the tailhead, inguinal area, and abdomen were immediately clipped and aseptically prepared. Enough adipose tissue to fill a 50-mL conical tube was harvested from the area lateral to the tailhead, the inguinal region, and the small colon mesentery by surgical excision. Additionally, adipose tissue was collected by lipoaspiration from the contralateral tailhead by making a 1-cm stab incision with a #15 scalpel blade 8 cm from midline adjacent to the tailhead. The adipose tissue was aspirated with a lipoaspirate cannula (3-mm Disposable Cannula; Shiplett Medical Technologies, Centennial, Colorado, USA) and a 60-mL luer lock syringe (VacLok; Merit Medical Systems, South Jordan, Utah, USA), without the use of tumescent fluid, to collect approximately 10 to 15 mL of adipose tissue.

Excisional adipose tissue samples from each location were divided into 2 portions, 1 for immunohistochemical (IHC) analysis and the other for cell isolation. The adipose for IHC was fixed in 10% buffered formalin. The lipoaspirate was evaluated for cell isolation only and not used for density or IHC evaluation.

#### Nucleated cell isolation and counting

The weight of each sample for cell isolation was determined before enzymatic processing. The lipoaspirates were processed in aliquots of approximately 10 g using a commercially available system (ARC System; InGeneron, Houston, Texas, USA) to isolate SVF cells from the adipose tissue. Lipoaspirates were incubated at 37°C and agitated in the processing unit with a proprietary blend.
of proteolytic enzymes (Matrase reagent; InGeneron) for 30 min to dissociate the tissue and release the nucleated cells, which were then separated from the solid tissue by filtration (100 μm filter, Steriflip; EMD Millipore, Billerica, Massachusetts, USA). The excisional adipose tissue samples used for cell isolation were minced with Mayo scissors and processed in approximately 10-gram aliquots. Similar to liposapirates, the minced excisional adipose samples were processed with Matrase reagent (InGeneron) at 37°C, with the exception that processing time was 60 min, following the manufacturer’s recommendation for excisional adipose tissue.

After processing and filtration, all samples were treated similarly by adding approximately 25 mL of Lactated Ringer’s solution (LRS) to reach a total volume of 50 mL to dilute enzymes and achieve balance and centrifuged for 10 min at 600 × g. The cells were washed twice by centrifugation and resuspension of the cell pellet in 40 mL of LRS each time. The final cell pellet was resuspended in 5 mL of LRS. Samples from each tissue site for each horse were run in duplicate.

To obtain nucleated cell counts, cells were stained with fluorescent nucleic acid stain (SYTO 13; Life Technologies/Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a 1:1 solution and then counted using a hemocytometer and standard light microscope at 40× magnification with a portable imaging cytometer (Bioscope; InGeneron). Non-viable cells were quantified by preparing a 1:1 solution of the cell suspension from each sample in 0.4% Trypan Blue solution. Non-viable cells were counted using a hemocytometer under light microscopy at 40× magnification. Nucleated cell viability was calculated using the following formula: (Nucleated Cells − Dead Cells)/Nucleated Cells × 100.

The remaining cells not used for counting were centrifuged at 600 × g for 10 min and the supernatant removed. The final stromal vascular fraction (SVF) was suspended in 2 mL of LRS and aliquots were mixed with an equal volume of Trizol reagent (TRIzol; Life Technologies/Thermo Fisher Scientific) to lyse cells and preserve the ribonucleic acid (RNA) and then frozen at −80°C until gene expression analyses in cell culture (CryoStor CS 5; BioLife Solutions, Bothell, Washington, USA).

### Adipose tissue density

The mass of each sample was determined using a digital electronic scale (PB303; Mettler-Toledo, Columbus, Ohio, USA) and the samples were then submerged in a known volume of deionized water at a temperature of 37°C in a graduated cylinder. A stylet from a 20-gauge, 8-in spinal needle was used to submerge the adipose tissue and this was used each time for consistency. The volume of water displaced by the sample was used to determine the density of the sample, using the formula Density = Mass/Volume.

### Immunohistochemistry

The fixed adipose tissues were embedded in paraffin and routinely sectioned at ~5 to 8 μm, then mounted on glass slides for IHC (immunohistochemistry) staining, which was done manually. A series of degrading alcohol steps was used to dehydrate the sections. The blocking step with 10% goat serum was carried out before incubation with primary antibody. The slides were incubated for 120 min with 1:100 rabbit anti-human polyclonal factor-VIII antibodies (Factor VIII; Dako North America, Carpenteria, California, USA), which have been shown to cross-react with equine tissue. After incubation, slides were rinsed and anti-rabbit mouse immunoglobulin G (IgG) secondary antibody (HK638-UK; BioGenex, Fremont, California, USA) was applied and allowed to incubate for 15 min. Horseradish peroxidase (Life Technologies/Thermo Fisher Scientific) was applied to tissue sections for 15 min. NovaRED peroxidase (Vector Laboratories, Burlingame, California, USA) was applied to the sections for 5 min and the sections were then washed with deionized water and counterstained with hematoxylin for 2 min. The factor VIII-stained adipose tissue was evaluated under light microscopy by 1 evaluator who was blinded (GM). The factor VIII-stained capillaries were manually counted at 40× power in 3 fields of view that were randomly selected.

### Quantitative PCR

The relative gene expression of MSC surface markers CD44, CD73, CD90, CD105, CD146, CD166, hematopoietic markers CD34 and CD45, endothelial cell marker CD31, along with genes associated with adipogenic (PPARG2), osteogenic (OSTEOCALCIN) lineages, as well as type-1 collagen (Col 1A2) was measured in total RNA from each adipose source.

For each condition tested, 2 replicates of total RNA were isolated from freshly isolated cells and measured using spectrophotometry (Nanodrop ND-100; Thermo Fisher Scientific, Wilmington, Delaware, USA). First strand complementary deoxyribonucleic acid (cDNA) synthesis (First Strand Kit; Qiagen, Chatsworth, California, USA) was carried out using 0.500 g of total RNA, according to the manufacturer’s instructions. All individual samples were analyzed in duplicate. Each plate contained both a no-reverse transcriptase (RT) control and no-template control and the gene used as constitutive baseline was β-actin. Quantitative PCR was carried out using a thermal cycler (iCycler; Bio-Rad, Hercules, California, USA) and a complete amplification mix (Green Supermix; Bio-Rad), with primer sequences listed in Appendix A (Sigma Genosys; Sigma-Aldrich, St. Louis, Missouri, USA).

### Colony-forming unit fibroblast assay

The assay for colony-forming unit fibroblasts (CFU-Fs) was adapted from an established method (20). Cells were plated and CFU-Fs were induced after first passage. For each adipose sample, cells from the first passage were plated at 3 cell densities: 250 000 cells, 50 000 cells, and 10 000 cells per 35-mm diameter well and repeated twice for each density. Cells were incubated for 14 d in standard growth media with 10% horse serum. Media were changed twice weekly. The cells were fixed with 4% formalin, stained with hematoxylin for 10 min, washed with phosphate-buffered saline (PBS), and pictures were obtained from 5 randomly chosen sections in each well for counts using a 2.5× objective on a light microscope. Two different experienced investigators, blinded to cell source, counted all visible colonies individually. A colony-forming unit was defined as a cluster containing at least 10 fibroblast-like, fusiform cells. After counting, the density of 10 000 cells per well was selected for further evaluation. The CFU-Fs were expressed as CFU-F% per 1 × 10⁶ adherent cells.
The source of the adipose resulted in significantly different nucleated cells/gram of tissue \( P \leq 0.0001 \) [mean values \( \pm \) standard error (SE): lipoaspiration \( = 761.667 \pm 227.348 \); tailhead \( = 1900.000 \pm 227.348 \); mesentery \( = 2.608.333 \pm 227.348 \); inguinal \( = 992.333 \pm 227.348 \)\] and percent viable \( P = 0.0150 \) (lipoaspiration \( = 86.8 \pm 3.3 \); tailhead \( = 92.5 \pm 3.3 \); mesentery \( = 96.8 \pm 3.3 \); inguinal \( = 91.1 \pm 3.3 \)\], but not for tissue density \( \left( g/cm^3 \right) \) \( P = 0.0666 \) [tailhead \( = 0.94 \pm 0.008 \); mesentery \( = 0.92 \pm 0.008 \); inguinal \( = 0.938 \pm 0.008 \)\] (Figure 1). The IHC

**Table I. The relative expression of each gene for the stromal vascular fraction (SVF) from each of the 4 sources**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Lipoaspiration</th>
<th>Tailhead</th>
<th>Mesentery</th>
<th>Inguinal</th>
<th>P-value</th>
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<tbody>
<tr>
<td>CD44</td>
<td>4.7a</td>
<td>3.66b,c</td>
<td>4.06b,c</td>
<td>3.78b,c</td>
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<td>CD73</td>
<td>7.02a</td>
<td>5.75b</td>
<td>5.21b</td>
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<tr>
<td>CD90</td>
<td>4.35a</td>
<td>2.87b,c</td>
<td>3.14b</td>
<td>2.25b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>CD105</td>
<td>4.16a</td>
<td>4.32a</td>
<td>4.89b,c</td>
<td>4.61b,c</td>
<td>0.007</td>
</tr>
<tr>
<td>CD146</td>
<td>4.5a</td>
<td>4.85a</td>
<td>5.79b,c</td>
<td>5.09b,c</td>
<td>0.0041</td>
</tr>
<tr>
<td>CD166</td>
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<td>6.26</td>
<td>6.23</td>
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<td>2.91a</td>
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<tr>
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<td>5.93a</td>
<td>5.06b</td>
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<td>2.29</td>
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<td>5.17</td>
<td>4.36</td>
<td>0.1934</td>
</tr>
<tr>
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<td>11.73</td>
<td>11.29</td>
<td>10.78</td>
<td>0.4789</td>
</tr>
<tr>
<td>Col1A2</td>
<td>2.07a</td>
<td>0.57b</td>
<td>3.7c</td>
<td>2.12a</td>
<td>&lt; 0.0001</td>
</tr>
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</table>

The qPCR data were normalized to \( \beta\)-actin and quantitated with the delta-delta cycle threshold (CT) method. As the data in the table are the expression of the gene relative to \( \beta\)-actin, for the CD44 lipoaspirate sample, the expression was 4.7 times that of the housekeeping gene \( \beta\)-actin. 

**Statistical analysis**

Data were entered into a spreadsheet (Microsoft Office Excel; Microsoft, Redmond, Washington, USA) and imported into a statistical software package for analysis (SAS; SAS Institute, Cary, North Carolina, USA). Responses were analyzed using linear-mixed models, with location and age as the fixed effects and horse as the random effect. For variables found to be significant in the model, comparisons among locations were assessed using an F-test, followed by Tukey pairwise comparisons. The qPCR data were normalized to \( \beta\)-actin and quantitated with the delta-delta cycle threshold (CT) method. There were 3 groups for comparison of density and immunohistochemistry (IHC), because the physical material of the lipoaspiration did not permit determination of these variables. The CFU-F data were evaluated with a 1-way analysis of variance (ANOVA) using tissue source as the main effect and a Tukey’s post-hoc test. A \( P \) of \( \leq 0.05 \) was considered significant.

**Results**

Figure 1. The mean (\( \pm \) SE) yield of nucleated cells/gram of tissue (A) and percent viability (B) for lipoaspirate, tailhead, inguinal, and mesenteric adipose. The density (C) and factor-VIII immunohistochemistry (IHC) (D) were obtained for tailhead, inguinal, and mesenteric adipose only because the lipoaspiration from the tailhead was represented by the solid sample from the tailhead and lipoaspiration was not physically conducive to obtaining these data. Columns with different superscript letters denote a significant difference.
showed a significantly different capillary density (capillaries/hpf) among locations, as shown by factor-VIII staining \( P < 0.0001 \) (tailhead = 20.6 ± 1.56; mesentery = 23.6 ± 1.56; inguinal = 10.6 ± 1.56) (Figure 1).

For the qPCR outcome data, adipose source was significantly related to differences found for CD 44 \( (P = 0.0095) \), CD 45 \( (P = 0.0006) \), CD 73 \( (P < 0.0001) \), CD 90 \( (P < 0.0001) \), CD 105 \( (P = 0.0007) \), CD 146 \( (P = 0.0041) \), CollA2 \( (P < 0.0001) \), and CD 34 \( (P = 0.0067) \) (Table 1). Osteocalcin was the only outcome variable significantly related to age \( (P = 0.048) \). The mean osteocalcin expression of the 15-year-old horse was 10.2 compared to 11.5 for all the remaining samples.

The CFU-F assay also resulted in significantly different frequencies of CFU-F formation among the various adipose sources. The mean \( (±\text{SD}) \) CFU-F% for the liposuction was 7.0% \( (±0.11) \) and for the mesentery was 8.59% \( (±0.11) \), both of which were significantly higher than the excisional adipose from the tailhead \( (2.42% \pm 0.04) \) and inguinal \( (2.11% \pm 0.06) \) locations (Figure 2).

A subjective finding not included as an outcome parameter but identified during the study was that adipose obtained by liposuction and from the mesentery could easily be filtered after enzymatic processing, whereas excisional adipose from the tailhead occasionally required a second filter because of clogging from fibrous tissue. All the inguinal samples required multiple filters, which suggests a denser connective tissue matrix.

**Discussion**

The data obtained in this study confirmed our hypothesis that the more vascular, dense adipose tissue would yield a higher concentration of nucleated cells. We found that there are significant differences among locations for nucleated cell yield per gram of adipose, viability, gene expression, and CFU-F%. We also found that fewer capillaries, as identified by factor-VIII IHC in the inguinal adipose, corresponded to the lowest yield of nucleated cells/gram, which is similar to results of previous studies (16).

The mesenteric adipose tissue provided the highest nucleated cells/gram of tissue and viability, which corresponds with the IHC vascular density findings among the tissue sites. The nucleated cells/gram in the excisional adipose from the tailhead were statistically similar to those in the mesentery and were significantly higher in both sites than in the lipos aspirate from the tailhead. The viability and yield of the lipos aspirated adipose tissue from the tailhead were the lowest of the 4 groups. Physical destruction during the lipospiration process can contribute to lower recovery and viability. It is well-established for human subcutaneous adipose tissue that harvest method can affect cell yield (21). The lower yield associated with mechanical trituration of adipose tissue caused by lipospiration has been previously described (18) and was similarly seen in this study.

We chose not to use tumescent fluid in this study because its analgesic properties were unnecessary, as the tissues were harvested from euthanized horses and we could accurately determine nucleated cells/gram of tissue. This could cause decreased yield and viability, however, as the tumescent fluid acts as a lubricant and buffer from physical damage during the aspiration process and speeds up the harvest of the adipose, which decreases the tissue damage created by the motion of the cannula. In a study comparing adipose collection techniques in humans, however, liposuction with tumescent fluid and adipose collected by resection were not significantly different in terms of viability and cellular yield of MSCs, while doubling times were significantly longer with ultrasound-assisted liposuction (22).

Tissue density was determined because it was initially thought that adipose with a greater percentage of adipose vacuoles and therefore a lower density would contain less vasculature and fewer nucleated cells. We found the opposite in that the tissue with the higher density had lower nucleated cells/gram because it was higher in dense fibrous connective tissue. Although the differences in densities were not significant, the mesenteric adipose tissue was much more easily digested and had less fibrous tissue. The lipos aspirated adipose tissue from the tailhead behaved similarly to the mesenteric adipose tissue during digestion and filtration. There was more connective tissue within the inguinal adipose during its harvest, mincing, and filtering. This was also evident histologically and corresponded with the higher density of the inguinal tissue. It is intuitive to think that the inguinal adipose tissue generated lower nucleated cells/gram due to the fibrous connective tissue, which would lower cellular yield.

It is known that most of the MSCs in bone marrow are in the initial segment of the collection and a small percentage of nucleated cells are MSCs (15,23). An advantage of any of the adipose sources is that there can be large deposits with a consistent yield of cells/gram of tissue that do not decline like bone marrow sources. In order to gain more MSCs from adipose, more adipose can be harvested and processed. The results of this study showed a similar cell yield for the lipos aspirate from the tailhead \( (7.65 \times 10^8) \) as in a previous study \( (7.9 \times 10^8) \) (18). The previous study demonstrated a 23-times increase in adherent cells and 50 times as many CFU-Fs compared to an equal number of nucleated cells derived from bone marrow.

The value of adipose-derived stromal vascular fraction (SVF) for regenerative medicine is emphasizing the need to further characterize the SVF. It is recognized that there are changes in surface markers between the SFV and cultured adipose-derived stem cells (24). The data presented here are the first data obtained from equine adipose and provide information about surface markers on the equine SVF that can be used in future studies. Differences in gene expression...
were found among the sources. The SVF from all tissue sources expressed CD73, CD90, and CD105 cell surface markers, which according to the International Society for Cellular Therapy, denotes the presence of MSCs (25). Although there is no specific surface marker group for equine stem cells, in those used for this study, the lipoaspirate sample showed the significantly highest expression for CD44, CD73, and CD90. This indicates that, despite somewhat lower cell yields, there may be a higher percentage of MSCs in the SVF obtained by lipoaspiration. The CFU-F data also demonstrated that there was a higher percentage of CFU-Fs in the lipoaspirate and the mesenteric samples.

Since these cells are from the SVF with multiple cell types present, we expected expression of hematopoietic markers (25). The hematopoietic markers were highest in the tailhead (CD45) and the mesentery (CD34) and lower in the inguinal region, similar to the IHC factor-VIII staining for capillaries. The niche concept, where stem cells are located close to the vasculature, is consistent with our findings (26,27). As the perivascular environment may provide the conditions that maintain the population of undifferentiated MSCs, the SVFs with the maximal MSC yield are likely to contain numerous cells positive for CD34 and CD45.

It is difficult to compare our results for gene expression with other studies of equine SVF because of the variation in isolation methods and cellular surface markers being studied. Some of the many types of cells present in the SVF can also stimulate healing, similar to MSCs. Thestromal vascular fraction contains fibroblasts, endothelial cells, leukocytes, and very small embryonic-like cells with additional cytokine production and healing capabilities that are not found in culture-expanded stem cells (28–30). In addition to the cells being transplanted when using the SVF, a recent study showed the ability of the SVF to induce or enhance MSC migration and proliferation in vitro (14). It was speculated that mediators released from the SVF resulted in the marked migration of the MSCs in the culture.

The objective of this study was to make a relative comparison of adipose depots for harvesting SVF in the horse using qPCR to determine the relative gene expression. While immunophenotyping of cells by flow cytometry could be complementary to the data obtained in this study, quantitative PCR was used in this study for a number of reasons. In studies where both methods have been used, outcomes have been similar (31,32). As there is no uniformly accepted definitive phenotype or surface marker for isolating MSCs from uncultured samples, they have been phenotypically identified retrospectively (33). Cell surface markers change with culture and at this time we do not have a composite phenotype of freshly isolated MSCs or a single marker that solely identifies native MSCs (34). In 1 study in which fresh bone marrow aspirate was followed over time, gene expression followed the same pattern as cellular protein expression at each time point (31). There are relatively few antibodies that have been validated in the horse and the relative expression of qPCR data has provided a viable comparison when antibodies have failed to bind equine cells (20,35).

Quantitative PCR has been used previously for cell surface markers of MSCs from multiple equine tissues (32,35–37). The expression of the surface markers CD90 and CD105 as characteristic of equine MSCs has also been determined by qPCR (37). In the heterogenous population of the SVF, the relative expression of the genes evaluated provides information about differences in the cell populations at the different locations. Because of potential issues with cellular debris and the multiple cell types present in fresh SVF, the question of the validity for multiple antibodies in the horse, and the lack of previous data using flow cytometry in fresh SVF of the horse, qPCR provided the most valuable information for this study.

The CFU-F is the standard for defining the number of progenitor cells in the SVF (24). The frequency of CFU-F from human adipose ranges from 1% to 10%. Our data from the horse fall into this range, with inguinal SVF at 2.11% and mesenteric SVF at 8.9%. The SVF from the mesentery and lipoaspirate was statistically similar and both were higher than that from the solid adipose from the tailhead and inguinal region. The mesenteric adipose had the highest cell yield, viability, and factor-VIII staining and the lowest density of CFU-Fs.

We selected locations that would represent abundant adipose depots and flexibility in harvesting during different recumbencies. The mesentery was included as a harvest site because many athletic horses have limited subcutaneous deposits. Furthermore, the other adipose tissues were of similar subcutaneous origin and the abdomen represented a source that may be expected to be different based on location and gross appearance. Mesenteric adipose can be harvested with the horse in dorsal recumbency during a surgical procedure when it is elected to obtain a SVF or it can be obtained in other horses with limited invasiveness by laparoscopy.

This study was designed to compare autogenous cell yield from different locations within a horse to determine if there is an optimum source. We did not select horses based on age, but there was a horse that was 15 y old in the study. No relationship was found between cell yield and age. It has been shown that age can affect the number of MSCs obtained from bone marrow, as the ilium is found to deplete with age in equids, which makes it an unreliable source of bone-marrow collection in older horses. In humans, the CFU-F numbers also significantly decrease in older age groups (38,39).

A potential limitation of this study is that the multipotency of the mesenchymal stem cells (MSCs) present in the stromal vascular fraction (SVF) was not evaluated. As previous studies verified the multipotency of adipose-derived MSCs (18), however, it was not pursued in this study. Additionally, a larger group of horses with a more extensive age range could allow the effect of age on MSC yield to be further evaluated.

In this study, significant differences were found in viability, cellular yield, vascular density, gene expression, and CFU-F among adipose tissue depots in the horse. The clinical application of this information indicates that there are multiple sources of adipose in horses that can be used to collect SVF. Further work is needed to determine whether the differences among adipose sources observed in this study are clinically relevant for in vitro treatment of musculoskeletal injuries in horses.

Acknowledgments

The authors thank Lisa Leake, Dr. Michael Coleman, and Maria P. Almario for their technical support. This study was supported in kind by Ingeneron, Inc.
**References**


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**Appendix A. The primer sequences utilized in this study.**

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<td>CCGCTCTATGAAACCGGTACCTG</td>
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**Gene**: Gene symbol of the gene.

**Forward 5’**: Forward primer sequence.

**Reverse 5’**: Reverse primer sequence.


Evaluation of hyaluronic acid, procollagen type III N-terminal peptide, and tissue inhibitor of matrix metalloproteinase-1 as serum markers of canine hepatic fibrosis

Jonathan A. Lidbury, Aline Rodrigues Hoffmann, Joanna K. Fry, Jan S. Suchodolski, Jörg M. Steiner

Abstract

The only way to diagnose hepatic fibrosis in dogs is by histological assessment of a liver biopsy specimen. As this technique is invasive and susceptible to sampling variation, serum biomarkers are used to detect hepatic fibrosis in humans. The objective of this study was to assess the utility of hyaluronic acid (HA), procollagen type III N-terminal peptide (PIIINP), and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) as serum markers of canine hepatic fibrosis. Serum samples were collected from 47 dogs with histologically confirmed hepatobiliary disease and 24 healthy dogs in order to measure concentrations of HA, PIIINP, and TIMP-1. Hepatic fibrosis was staged using a 5-point scoring scheme. There was no correlation between serum concentrations of HA or PIIINP and the severity of hepatic fibrosis. There was a negative correlation between serum concentration of TIMP-1 and the severity of hepatic fibrosis ($r_s = -0.33; P = 0.036$). It was not possible to use serum concentrations of HA, PIIINP, or TIMP-1 to discriminate between dogs with absent-to-moderate hepatic fibrosis and those with marked-to-very-marked fibrosis. The results of this study do not support the utility of measuring serum concentrations of HA, PIIINP, or TIMP-1 for diagnosing canine hepatic fibrosis. Further studies are needed to support this finding.

Résumé

Le seul moyen de diagnostiquer la fibrose hépatique chez les chiens est par évaluation histologique d’un échantillon de biopsie hépatique. Étant donné que cette technique est invasive et sujette à variation dans l’échantillonnage, chez l’humain des marqueurs sériques sont utilisés pour détecter la fibrose hépatique. L’objectif de la présente étude était d’évaluer l’utilité de l’acide hyaluronique (AH), du peptide N-terminal du pro-collagène de type III (PNPIII), et de l’inhibiteur tissulaire de la métalloprotéinase-1 matricielle (ITMP-1) comme marqueurs sériques de la fibrose hépatique canine. Des échantillons de sérum ont été récoltés de 47 chiens avec une pathologie hépatobiliaire confirmée histologiquement et 24 chiens en santé afin de mesurer les concentrations d’AH, PNPIII, et ITMP-1. La fibrose hépatique a été catégorisée en utilisant un schéma de pointage en cinq points. Il n’y avait pas de corrélation entre les concentrations sériques d’AH ou de PNPIII et la sévérité de fibrose hépatique. Il y avait une corrélation négative entre la concentration sérique d’ITMP-1 et la sévérité de la fibrose hépatique ($r_s = -0.33; P = 0.036$). Il n’était pas possible d’utiliser les concentrations sériques d’AH, de PNPIII, ou d’ITMP-1 afin de différencier les chiens avec une fibrose hépatique absente à modérée de ceux avec une fibrose marquée à très marquée. Les résultats de la présente étude ne permettent pas de justifier l’utilité de mesurer les concentrations sériques d’AH, de PNPIII, ou d’ITMP-1 pour le diagnostic de la fibrose hépatique canine. Des études supplémentaires sont requises pour soutenir cette trouvaille.

(Traduit par Docteur Serge Messier)

Introduction

The development of hepatic fibrosis is an important event in the progression of liver disease and has been shown to be a negative prognostic factor in humans with chronic hepatitis (1). Currently, the only way to diagnose hepatic fibrosis in dogs is by histological assessment of a liver biopsy specimen. Liver biopsy is invasive, there is a risk of hemorrhage, and as only a small portion of the organ is evaluated (2); this technique is susceptible to sampling variation (3,4). Because of these disadvantages, serum markers of hepatic fibrosis have been developed for use in humans (5).

In human patients with hepatic fibrosis, an increased rate of extracellular matrix turnover results in matrix components being released into the bloodstream (5). Hyaluronic acid (HA) is a highly...
evolutionarily conserved glycosaminoglycan component of the extracellular matrix (6) that is used as a serum biomarker of hepatic fibrosis (5). In a study of humans with chronic hepatitis-C, the sensitivity and specificity of HA for distinguishing between patients with extensive fibrosis and those with milder fibrosis were 86% and 70%, respectively (7). It has previously been shown that HA increased in dogs with hepatic disease, including cirrhosis (8,9) and congenital portosystemic shunts (CPSS) (10).

In a study of human patients with chronic hepatitis-C, measurement of serum concentrations of procollagen type III N-terminal peptide (PIIINP) had a sensitivity of 92% and a specificity of 76% for differentiating between patients with extensive fibrosis and those with milder fibrosis (7). Serum concentrations of PIIINP were increased in growing dogs, but not in dogs with chronic bronchopulmonary disease (11). Serum concentrations of PIIINP have not previously been assessed in dogs with hepatobiliary disease.

Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) is a protein that inhibits the action of matrix metalloproteinase-1, thus slowing the degradation of extracellular matrix during fibrosis (12). In a study of human patients with chronic hepatitis-C, measurement of serum concentration of TIMP-1 had a sensitivity of 75% and a specificity of 70% for differentiating between patients with extensive fibrosis and those with milder fibrosis (7). To the authors’ knowledge, serum concentrations of TIMP-1 have not previously been assessed in dogs with hepatobiliary disease.

We hypothesized that serum concentrations of HA, PIIINP, and TIMP-1 would be positively correlated with the histological stage of hepatic fibrosis and would allow the discrimination between dogs with absent-to-moderate hepatic fibrosis and those with marked-to-very-marked fibrosis. The main objectives of this initial exploratory study were to determine if there is a correlation between serum concentrations of these analytes and the histological severity of hepatic fibrosis and to determine if these markers allow the discrimination between dogs with absent-to-moderate hepatic fibrosis and those with marked-to-very-marked fibrosis. The secondary objectives of the study were to compare serum concentrations of HA, PIIINP, and TIMP-1 among dogs with different types of hepatobiliary disease and healthy dogs, as well as to partially validate a nonspecies-specific serum HA assay for use with canine serum.

**Materials and methods**

**Animals**

Dogs over 1 y of age (over 2 y for large and giant breeds) with histologically confirmed hepatobiliary disease diagnosed at Gulf Coast Veterinary Specialists or Texas A&M University Veterinary Medical Teaching Hospital from March 1, 2011 to February 28, 2013 were enrolled in this prospective observational study. This age cutoff was used to avoid enrolling growing dogs. Diagnosis of hepatobiliary disease was based on a combination of clinical signs, laboratory testing, diagnostic imaging findings, histological evaluation of a liver biopsy specimen, and in some cases, findings after surgical exploration of the abdominal cavity.

These dogs were divided into 4 groups: i) chronic hepatitis; ii) hepatic neoplasia, which could be primary or secondary; iii) congenital portosystemic shunt (CPSS); and iv) other hepatobiliary diseases, including vascular hepatopathy, nodular regeneration, and gallbladder mucocele. When possible, an extra liver biopsy specimen was collected from the dogs with hepatobiliary disease for evaluating the severity of fibrosis.

Healthy staff-owned dogs over 1 y of age (over 2 y of age for large and giant breeds) were enrolled at the Texas A&M University Veterinary Medical Teaching Hospital. The health of these dogs was assessed by an owner questionnaire, physical examination, complete blood (cell) count (CBC), serum biochemistry profile, and measurement of serum concentration of pancreas-specific lipase (Spec cPL Test; IDEXX Laboratories, Westbrook, Maine, USA). Dogs with clinically important abnormalities were excluded from the study.

The study was approved by the Texas A&M University Institutional Animal Use Committee. Informed owner consent was given before the dogs were enrolled in the study.

**Serum samples**

At the time of liver biopsy or immediately before euthanasia and necropsy, 3 to 5 mL of blood were collected from the dogs by jugular venipuncture and placed into sterile anticoagulant-free tubes. Once a firm blood clot had formed, the blood was centrifuged at 1300 × g for 15 min to separate the serum from the red blood cells. The serum was stored at −80°C until analysis.

**Assays**

The assays were conducted in batches containing samples from healthy and diseased dogs. Samples were run in duplicate using materials, including standards and quality controls, provided by the respective manufacturers according to their instructions. Serum concentrations of HA were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) marketed for use in humans and other animals (Hyaluronan ELISA Kit; Echelon Biosciences, Salt Lake City, Utah, USA). This assay has previously been used for measuring HA in canine serum (13–16). Assay precision was assessed by calculating the intra-assay coefficients of variation (%CV) for 3 samples (low, medium, and high concentration) run 6 times on the same ELISA plate. Repeatability was assessed by calculating the inter-assay %CV for 3 samples (low, medium, and high concentration) run 7 times on different days. We assessed analytical accuracy by calculating observed-to-expected ratios (%) when equal volumes of 2 of 4 canine serum samples were mixed in every possible combination. Assay linearity was assessed by calculating observed-to-expected ratios (%) when 5 samples were serially diluted, 1:2, 1:4, 1:8, and 1:16, with sample buffer. Serum concentrations of PIIINP were measured with a human radioimmunoassay (UniQ PIIINPRIA; Orion Diagnostica, Espoo, Finland) that has previously been validated for use with canine serum (11). Serum concentrations of TIMP-1 were measured using a commercially available canine ELISA (TIMP-1 ELISA; USCN Life Science, China) that the manufacturer has validated for use with canine serum.

**Histological assessment**

Liver biopsies were fixed in neutral buffered formalin, processed for routine histology, and embedded in paraffin. Serial sections of liver were stained with hematoxylin and eosin and picrosirius red
(Picosirius Red Stain Kit; Polysciences, Warrington, Pennsylvania, USA). Hepatic fibrosis was staged by a board-certified veterinary anatomic pathologist (ARH) using a 5-point scoring scheme, i.e., 0 — absent, 1 — mild, 2 — moderate, 3 — marked, and 4 — very marked. This scheme is an adaptation of the Ishak scheme and was devised by Drs. van den Ingh, Grinwis, and Rothuizen at the University of Utrecht and has previously been used to assess hepatic fibrosis in dogs (17). Information about the clinical history or serum concentrations of extracellular matrix components was not provided to the pathologist during the scoring process.

**Statistical methods**

The distribution of continuous data was assessed using the Kolmogorov-Smirnov test and by visual inspection of frequency histograms. Non-parametric data were expressed as the median (minimum—maximum). Serum concentrations of HA, PIIINP, and HA were compared among the groups of dogs using the Kruskal-Wallis test, followed by post-testing with Dunn’s test as needed. Comparisons of continuous variables between 2 groups of dogs were made using Mann-Whitney U-tests. The relationships between serum HA, PIIINP, and TIMP-1 concentrations and the hepatic fibrosis stage were assessed using Spearman’s rank correlation ($r$). A statistical software package was used for these calculations (GraphPad Prism 5; GraphPad Software, LaJolla, California, USA). Statistical significance was set as $P < 0.05$.

**Results**

Forty-seven dogs with hepatobiliary disease were enrolled in the study. The median age of the dogs was 10 y (range: 1 to 17 y). Twenty were neutered males (43%), 3 were intact males (6%), 22 were spayed females (47%), and 2 were intact females (4%). The following breeds were represented: 3 Labrador retrievers (6%), 3 miniature schnauzers (6%), 3 Yorkshire terriers (7%), 2 Chihuahuas (4%), and 16 mixed-breed dogs (32%). Twenty dogs (43%) had chronic hepatitis, 17 (36%) had hepatobiliary neoplasia (13 with hepatocellular carcinoma/adenoma, 2 with hemangiosarcoma, 1 with cholangiocarcinoma, and 1 with lymphoma), 4 dogs (9%) had CPSS, and 6 (13%) had other hepatobiliary disease (2 dogs with gall bladder mucoceles, 2 with nodular hyperplasia, and 1 with portal vein hypoplasia and portal hypertension, and 1 with acute hepatitis). Twenty-four healthy dogs were enrolled in the study. The median age of these dogs was 3 y (range: 1 to 13 y). Eight were neutered males (33%), 11 were spayed females (46%), and 5 were intact females (21%). The following breeds were represented: 3 miniature schnauzers (13%), 2 German shepherds (8%), 2 hounds (8%), 2 Australian shepherds (8%), and 3 mixed-breed dogs (13%).

All the hepatic samples were deemed to be adequate for evaluation. Hepatic fibrosis stage scores were assigned to the dogs as follows: 6 dogs (13%) had no fibrosis, 10 dogs (21%) had mild, 14 dogs (30%) had moderate, 8 dogs (17%) had marked, and 9 dogs (19%) had very marked fibrosis.

The intra-assay %CV for the HA ELISA was 6.2%, 1.9%, and 12.2% for low, medium, and high concentration samples, respectively. The inter-assay variability %CV for the assay was 15.1%, 13.4%, and 15.3% for low, medium, and high concentration samples, respectively. The mean (% standard deviation; minimum—maximum) observed-to-expected ratio for spiking recovery experiments was 97.2% (± 7.2%; range: 89.5% to 110.9%). The mean observed-to-expected ratio for the dilutional parallelism experiments was 96.0% (± 16.9%; range: 75.4% to 129.3%).

 Serum concentrations of HA were measured in 45 dogs with hepatobiliary disease (96%) and in 24 healthy dogs (100%). There was no correlation between serum concentration of HA and the severity of hepatic fibrosis ($r_s = 0.24; P = 0.114$; Figure 1). There was no significant difference in serum concentrations of HA between dogs with absent-to-moderate hepatic fibrosis and those with marked-to-very-marked fibrosis ($P = 0.598$; Table I). Serum concentrations of HA were higher in healthy dogs (median: 201 ng/mL; range: 84 to 1464 mg/mL) than in dogs with neoplasia (median: 126 ng/mL; range: 82 to 1532 ng/mL; $P < 0.05$; Table I). There were no other significant differences in serum concentrations of HA among the groups of dogs (Table II).

 Serum concentrations of PIIINP were measured in 39 dogs with hepatobiliary disease (83%) and in 24 healthy dogs (100%). There was no correlation between serum concentration of PIIINP and the severity of hepatic fibrosis ($r_s = 0.12; P = 0.472$; Figure 2). There was no significant difference in serum concentrations of PIIINP between dogs with absent-to-moderate hepatic fibrosis and those with marked-to-very-marked fibrosis (Table II; $P = 0.707$). There were no significant differences in serum concentrations of PIIINP among the groups of dogs ($P = 0.440$; Table I).

 Serum concentrations of TIMP-1 were measured in 41 dogs with hepatobiliary disease (87%) and in 24 healthy dogs (100%). There was a negative correlation between serum concentration of TIMP-1 and the severity of hepatic fibrosis ($r_s = -0.33; P = 0.036$; Figure 3). There was no significant difference in serum concentrations of TIMP-1 between dogs with absent-to-moderate hepatic fibrosis and those with marked-to-very-marked fibrosis ($P = 0.124$; Table I). There were
Discussion

The HA ELISA that we used in this study had acceptable precision, accuracy, and linearity for measuring canine serum concentrations of HA. The inter-assay %CVs of 15.1%, 13.4%, and 15.3% were slightly higher than desirable, which suggests suboptimal repeatability. We do not think this is likely to have affected our conclusions, however, as there was no trend for serum concentrations of HA to be related to the severity of hepatic fibrosis. The relevance of the finding that dogs with hepatic neoplasia had lower serum concentrations of HA than healthy dogs is unknown and neither is the reason that this occurred. There were only small differences in the median concentration of HA between the groups, however, and it may therefore not be clinically important. We are not aware of a similar relationship in humans with hepatic neoplasia. There was no relationship between the severity of hepatic fibrosis and serum concentrations of HA in the dogs enrolled in this study. This is in contrast to studies in humans in whom HA has been shown to be a useful marker of hepatic fibrosis (5,7) and a previous study in dogs in which those with hepatic cirrhosis had higher serum concentrations of HA than those with non-cirrhotic hepatic disease, those with non-hepatic disease, or healthy dogs (8).

Another study also showed that serum concentrations of HA were higher in dogs with advanced hepatic fibrosis than in those with mild fibrosis (9). The aforementioned studies in dogs used a different assay than the one we used. Previous studies using the ELISA that we used in our study, however, found that Chinese Shar pei dogs with cutaneous mucinosis have higher serum concentrations of HA than healthy dogs or Chinese Shar peis without cutaneous mucinosis (13–16), which suggests that this assay is capable of detecting elevated serum concentrations of HA in dogs. The median serum concentrations of HA in 5 healthy dogs reported in 1 of these studies was 244 ng/mL (range: 166 to 302 ng/mL), which is similar to that of the 23 healthy dogs from our study with 201 ng/mL (range: 84 to 1464 ng/mL) (15). Another possible reason for the discrepancy

Table I. Serum concentrations of extracellular matrix components in dogs at different stages of hepatic fibrosis

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Absent-to-moderate fibrosis</th>
<th>Marked-to-very-marked fibrosis</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA concentration</td>
<td>1310 ng/mL (50 to 1532)</td>
<td>156 ng/mL (50 to 3360)</td>
<td>0.598</td>
</tr>
<tr>
<td>PIIINP concentration</td>
<td>8.2 µg/L (3.3 to 50.0)</td>
<td>9.8 µg/L (3.1 to 38.6)</td>
<td>0.707</td>
</tr>
<tr>
<td>TIMP-1 concentration</td>
<td>30 ng/mL (5 to 100)</td>
<td>19 ng/mL (6 to 59)</td>
<td>0.124</td>
</tr>
</tbody>
</table>

HA — Hyaluronic acid; PIIINP — Procollagen type III N-terminal peptide; TIMP-1 — Tissue inhibitor of matrix metalloproteinase-1.

Table II. Serum concentrations of extracellular matrix components in healthy dogs and dogs with various types of hepatobiliary disease

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Healthy</th>
<th>Chronic hepatitis</th>
<th>Hepatobiliary neoplasia</th>
<th>Congenital portosystemic shunt</th>
<th>Other hepatobiliary disease</th>
<th>P-value (Kruskall-Wallis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA concentration</td>
<td>201 ng/mL* (84 to 1464)</td>
<td>154 ng/mL (50 to 3360)</td>
<td>126 ng/mL* (82 to 1532)</td>
<td>171 ng/mL (70 to 576)</td>
<td>126 ng/mL (106 to 304)</td>
<td>0.038</td>
</tr>
<tr>
<td>PIIINP concentration</td>
<td>10.0 µg/L (3.8 to 48.2)</td>
<td>8.2 µg/L (3.1 to 38.6)</td>
<td>8.1 µg/L (3.3 to 35.3)</td>
<td>12.5 µg/L (6.0 to 16.3)</td>
<td>10.9 µg/L (3.3 to 22.7)</td>
<td>0.440</td>
</tr>
<tr>
<td>TIMP-1 concentration</td>
<td>20 ng/mL (5 to 100)</td>
<td>21 ng/mL (6 to 156)</td>
<td>45 ng/mL (6 to 100)</td>
<td>26 ng/mL (13 to 34)</td>
<td>23 ng/mL (5 to 63)</td>
<td>0.139</td>
</tr>
</tbody>
</table>

*P < 0.05 for post-testing between groups.

HA — Hyaluronic acid; PIIINP — Procollagen type III N-terminal peptide; TIMP-1 — Tissue inhibitor of matrix metalloproteinase-1.

no significant differences in serum concentrations of TIMP-1 among the groups of dogs (P = 0.139; Table II; Figure 4). Post-hoc analysis revealed that there was no significant difference in serum concentration of TIMP-1 between dogs with hepatic neoplasia (median: 45 ng/mL; range: 6 to 100 ng/mL) and dogs with non-neoplastic hepatobiliary disease (median: 22 ng/mL; range: 5 to 156 ng/mL; P < 0.1 but > 0.05). Post-hoc analysis showed that dogs with primary hepatobiliary neoplasia (hepatocellular adenomas, hepatocellular carcinomas, and cholangiocarcinomas) had higher serum concentrations of TIMP-1 than those with tumors and those with neoplasia secondarily affecting the liver (lymphoma and hemangiosarcoma), with median concentrations of 47 ng/mL (range: 25 to 100 ng/mL) and 6 ng/mL (range: 6 to 12 mg/mL), respectively (P = 0.0115).
in results between our study and these previous studies on dogs with hepatic disease (8,9) is that our study contained relatively few dogs with marked or very marked hepatic fibrosis and potentially serum concentrations of HA only increase in dogs with very marked fibrosis (n = 9).

There was no difference in serum concentrations of PIIINP among healthy dogs, those with chronic hepatitis, CPSS, hepatobiliary neoplasia, or other hepatobiliary disease. Additionally, no relationship was observed between the severity of hepatic fibrosis and serum concentrations of PIIINP. In humans, serum concentrations of PIIINP have been shown to be useful in distinguishing between patients with no or mild fibrosis and those with moderate or severe fibrosis (5,7). It is not known why serum concentrations of PIIINP were not increased in these dogs with hepatic fibrosis. One possible explanation is that this protein is not leaked into the bloodstream of dogs with hepatic fibrosis. Type III collagen was increased in the livers of dogs with chronic hepatitis (18), which suggests that this form of collagen is important in canine hepatic fibrosis. It is interesting that a previous study did not find that serum concentrations of PIIINP were increased in dogs with chronic bronchopulmonary disease, but that concentrations in bronchoalveolar lavage fluid were increased (11). In humans, PIIINP has also been shown to have a moderately strong positive correlation with the severity grade of hepatic necroinflammatory activity (19). We therefore cannot rule out that this was a confounding factor that obscured the presence of a true relationship between PIIINP and the severity of hepatic fibrosis in our study.

Upon visual inspection of the data, the median serum concentration of TIMP-1 in dogs with hepatic neoplasia (median: 45 ng/mL;
range: 6 to 100 ng/mL) was higher than that of healthy dogs (median: 20; range: 5 to 100 ng/mL) or a combined group of dogs with non-neoplastic hepatobiliary disease formed post-hoc (median: 22; range: 5 to 156 ng/mL), but the differences were not statistically significant. This was possibly due to type-II error, as the sample size in each group was relatively small. Additionally, dogs with primary liver neoplasia (hepatocellular adenoma/carcinoma and cholangio carcinoma) had significantly higher serum concentrations of TIMP-1 than those with lymphoma and hemangiosarcoma.

Humans with a variety of tumors, including hepatic metastases (20) and pulmonary neoplasms (21), have been shown to have increased serum concentrations of TIMP-1. In 1 study of humans with hepatic metastases, higher serum concentrations of TIMP-1 were shown to be a poor prognostic factor (20). Dogs with spontaneously occurring mammary tumors have been shown to have a relatively low tissue TIMP-1 activity compared to rats with induced mammary tumors (22), but, to our knowledge, serum concentrations of TIMP-1 have not previously been reported in dogs with hepatobiliary neoplasia. It is important to emphasize, however, that it was not hypothesized a priori that serum concentrations of TIMP-1 would be higher in dogs with primary hepatic neoplasia than in metastatic tumors. This means that we cannot draw any definitive conclusions from this finding. Further studies in a larger group of dogs with hepatic neoplasia are needed to confirm or refute our findings and to assess serum concentrations of TIMP-1 in dogs with other types of neoplasia.

There was a weak negative correlation between serum concentration of TIMP-1 and the severity of fibrosis. As there was no significant difference in the fibrosis stages assigned to dogs with hepatobiliary neoplasia and those with other hepatobiliary diseases, it is unlikely that the non-significant trend for increased serum concentrations of TIMP-1 observed in dogs with neoplasia acted as a confounding factor, which explains this unexpected negative correlation. There was no difference in serum concentrations of TIMP-1 between dogs with absent-to-moderate fibrosis and those with marked-to-very-marked fibrosis. These findings contradict those in studies of humans in which TIMP-1 is a useful marker of hepatic fibrosis and is positively correlated with its severity (5,7). The reason for this difference between the 2 species is not known.

It is important to discuss several limitations of this work. Firstly, as there were relatively few dogs (9 out of 48; 19%) that were assigned a score of very marked hepatic fibrosis, it is possible that this was why we failed to find a relationship between any of the extracellular matrix components and the severity of hepatic fibrosis because they are only increased in dogs with very marked fibrosis. Even if this was the case, however, the utility of these markers would be limited if they can only distinguish between dogs with mild fibrosis and those with very marked fibrosis. The sample sizes in some of the groups were small and this may have led to type-II error when evaluating differences in the analytes between these groups. Additionally, we cannot completely exclude the possibility that some of the dogs that were enrolled in this study had subclinical disease that was causing fibrosis of another organ, therefore increasing their serum concentration of extracellular matrix components and acting as a confounding factor. The group of dogs with hepatobiliary disease in this study, however, would be similar to the population of dogs in which these markers would be used if they were shown to be valuable. It is therefore important to recognize their lack of apparent diagnostic utility in this population.

In conclusion, serum concentrations of HA, PIIINP, and TIMP-1 were not able to discriminate between dogs with and without hepatic fibrosis. Therefore, the results of this study do not support the utility of measuring serum concentrations of HA, PIIINP, or TIMP-1 for diagnosing canine hepatic fibrosis. Further studies are needed to confirm this finding.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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13. Grützner N, Heilmann RM, Cranford SM, Holzenburg A, Suchodolski JS, Steiner JM. Inflammatory, immunological,


Identification and characterization of pig adipose-derived progenitor cells

Shuang Zhang, Chunyu Bai, Dong Zheng, Yuhua Gao, Yanan Fan, Lu Li, Weijun Guan, Yuehui Ma

Abstract

Adipose-derived stem cells (ADSCs) are multipotent, and can be differentiated into many cell types in vitro. In this study, tissues from pigs were chosen to identify and characterize ADSCs. Primary ADSCs were sub-cultured to passage 28. The surface markers of ADSCs: CD29, CD71, CD73, CD90, and CD166 were detected by reverse-transcription polymerase chain reaction assays and the markers CD29, CD44, CD105, and vimentin were detected by immunofluorescence. Growth curves and the capacity of clone-forming were performed to test the proliferation of ADSCs. Karyotype analysis showed that ADSCs cultured in vitro were genetically stable. To assess the differentiation capacity of the ADSCs, cells were induced to differentiate into osteoblasts, adipocytes, epithelial cells, neural cells, and hepatocyte-like cells. The results suggest that ADSCs from pigs showed similar biological characteristics with those separated from other species, and their multi-lineage differentiation shows potential as an application for cellular therapy in an animal model.

Introduction

Adipose tissue is an abundant, less expensive, and safe bodily tissue to study. It can be isolated in large quantities by minimally invasive liposuction. Immature pluripotent cells are important targets for tissue engineering, regenerative medicine, and gene therapy (1). Adipose-derived stem cells (ADSCs) are the richest sources of multipotent cells and can be easily cultivated and expanded. These cells pose less psychological and physical impact to the donor than bone marrow cells (BMCs). The phenotype and expansive capacity of ADSCs are stable, without spontaneous re-differentiation and shift of cellular markers after dozens of passages (2). They have a high capacity to differentiate and self-renew within and across lineage barriers (3). Adipose-derived stem cells can be differentiated into osteogenic, adipogenic, myogenic, chondrogenic, and neurogenic lineages (3–5). Adipose-derived stem cells also have low immunogenicity, which offers great promise for use in regenerative medicine (6–8). Other research shows ADSCs represent a practical, abundant, and appealing source for cell replacement of donor tissue (9).

Currently, the shortage of available donors and graft rejection hamper widespread transplantation. Stem cell therapy is a current research topic and holds great promise for disease treatment. Adipose-derived stem cells can grow rapidly in vitro. They are available for harvesting in large numbers using a less invasive operation. In this research, we describe the isolation and culture procedures of pig ADSCs, and demonstrate that ADSCs are able to differentiate into many cell types.

Materials and methods

Isolation, culture, and purification of ADSCs

Animal experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Chinese Academy of Agricultural Sciences. Adipose tissues were harvested from the visceral (omentum) region of pigs under aseptic conditions. Briefly, the extracellular matrix was dissociated with 0.1% (m/v) type I collagenase (Sigma) and the harvested...
pellet was re-suspended in complete medium (10). At 70% to 80% confluence, the cells were passaged with 0.125% trypsin. Generally, after 3 to 4 passages, the cells were homogenous.

### Identification of ADSCs

**Surface marker detection.** Cells were incubated in the following antibodies: i) mouse anti-pig CD29 (1:100; Abcam); ii) rat anti-pig CD44 (1:100; Abcam); iii) mouse anti-pig CD105 (1:100; Abcam); and iv) mouse anti-pig Vimentin (1:100; Abcam). The cells were then incubated by FITC-conjugated goat anti-mouse immunoglobulin and goat anti-rat immunoglobulin (bioss) and examined using a Nikon TE-2000-E confocal microscope.

**RNA isolation and RT-PCR.** RNA was extracted from the cells from passages 4, 14, 20, and 26 using Trizol reagent (Invitrogen).

### Table I. Primer information for adipose-derived stem cell identification

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
<th>Circles</th>
<th>Product length (bp)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD29</td>
<td>F 5’ TGATTGCTTGGTTCTACTCTACA 3’ R 5’ TTCCCTCATACTCCTGGATTGAC 3’</td>
<td>30</td>
<td>302</td>
<td>58</td>
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<tr>
<td>CD71</td>
<td>F 5’ CATGCAAACTGGTTCTCTCA 3’ R 5’ TCCTGGAAGGTCTCATTAGG 3’</td>
<td>30</td>
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<tr>
<td>CD73</td>
<td>F 5’ AACCCACCTTCCAAGAGGT 3’ R 5’ GTGCCATCAGTCAGTACGG 3’</td>
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<tr>
<td>CD90</td>
<td>F 5’ GGCATGCTCTCTTTCTAAAC 3’ R 5’ GGACCTTGATGTCGTACTTGC 3’</td>
<td>30</td>
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<tr>
<td>CD166</td>
<td>F 5’ CCATCACTGTTCTCATTG 3’ R 5’ GCGGAGCAGTGGTACAGG 3’</td>
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<td>CD31</td>
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<td>COL1al</td>
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<td>30</td>
<td>207</td>
<td>63</td>
</tr>
<tr>
<td>RUNX2</td>
<td>F 5’ GAGAGAGAGAGAGAGAGAGAGGA 3’ R 5’ TGTTTGTGAGGCGAATGAAG 3’</td>
<td>30</td>
<td>206</td>
<td>57</td>
</tr>
<tr>
<td>OPN</td>
<td>F 5’ TTCCCAAGTCAGCCCAAGATT 3’ R 5’ TTGGTTCAGACACACACAG 3’</td>
<td>30</td>
<td>262</td>
<td>60</td>
</tr>
<tr>
<td>LPL</td>
<td>F 5’ TGACAAAGATGGCCAGTGCT 3’ R 5’ TTCCCAAGGACAGAATGC 3’</td>
<td>30</td>
<td>208</td>
<td>59</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>F 5’ CCAGGTCTTGTGTAAGTTGA 3’ R 5’ TAGAGTGGTGAGGCTCA 3’</td>
<td>30</td>
<td>216</td>
<td>58</td>
</tr>
<tr>
<td>ALB</td>
<td>F 5’ GCCTTGTGTTGAGCCTCA 3’ R 5’ GTCCAGACCAGGGACAGAT 3’</td>
<td>30</td>
<td>241</td>
<td>62</td>
</tr>
<tr>
<td>AFP</td>
<td>F 5’ GCCAAAAGTTGAGGAGGAGA 3’ R 5’ AGCACCGAGAAAGAATCG 3’</td>
<td>30</td>
<td>143</td>
<td>60</td>
</tr>
<tr>
<td>MAP2</td>
<td>F 5’ TCAATGCAACGAGCATACGC 3’ R 5’ TTCTTCCTGCCCCTTTTT 3’</td>
<td>30</td>
<td>398</td>
<td>58</td>
</tr>
<tr>
<td>NF</td>
<td>F 5’ GGGAAGGTGGTGGAGGAAGA 3’ R 5’ GGGACATGGGGGATG 3’</td>
<td>30</td>
<td>460</td>
<td>56</td>
</tr>
<tr>
<td>CK18</td>
<td>F 5’ TGCAATACTGTTGGACATG 3’ R 5’ TCTTCTGGGTTCTCTGACG 3’</td>
<td>30</td>
<td>375</td>
<td>60</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>F 5’ CCCAACCTTCCCTCCTC 3’ R 5’ CACCCCTTCTCCTCAGAAATA 3’</td>
<td>30</td>
<td>281</td>
<td>60</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F 5’ ACCCAGAAGAGACTGTGGAAGG 3’ R 5’ CTAGTGTAGCAGCCAGATGC 3’</td>
<td>30</td>
<td>285</td>
<td>60</td>
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Figure 1. Morphology and characteristics of adipose-derived stem cells (ADSCs). A — Morphology of primary cultured and sub-cultured adipose-derived progenitor cells. (a) Primary cells after culture for 48 h. Many cells started to adhere. (b) ADSCs exhibited a fibroblast-like morphology and expanded easily. (c) ADSCs grew to 90% confluence about 7 days later. (d) The ADSCs of passage 28 displayed cell senescence. Scale bar = 50 μm.

B — Characteristics of ADSCs surface antigens at different passages. Immunofluorescence showing CD29-, CD44-, CD105- and vimentin-positive cells. Scale bar = 50 μm.

C — RT-PCR analysis showed that ADSCs at different passages express CD29, CD71, CD73, CD90, and CD166, while they didnot-positive cells Lanes 1 CD29, 2 CD71, 3 CD73, 4 CD90, 5 CD166, 6 CD31, 7 GAPDH. GAPDH served as the internal control.
We used the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 for reverse-transcription polymerase chain reaction (RT-PCR). The cDNAs were amplified by Emerald Amp Max PCR (TaKaRa). The specific primers are listed in Table I. The PCR products were visualized by 2% agarose gel electrophoresis.

Growth kinetics

To assess growth dynamics, ADSCs at different passages were seeded in triplicate in 24-well plates (1 × 10^4 cells/well) (11). The population doubling time (PDT) was calculated as follows:

\[ \text{PDT} = \frac{(t - t_0) \log_2}{\log N_t - \log N_0}, \]

Where: \( t_0 \) = start time of culture, \( t \) = termination time of culture, \( N_0 \) = initial number of cells in culture, and \( N_t \) = the final number of cells in culture.

Colony-forming cell assay

Cells from passages 4, 12, and 27 were seeded in 24-well microplates at a density of 1 × 10^4 cells/well, and numbers of colony-forming units (CFU) were counted to calculate the colony-forming rate, which is formulated as CFU numbers/starting cell number per 24-well × 100%.

Karyotype analysis

The karyotype of P8 cells was analyzed. Cells were subjected to hypotonic treatment and fixed, and the chromosome numbers were counted from 100 spreads under an oil immersion objective upon Giemsa staining.

Table II. Medium information for adipose-derived stem cells

<table>
<thead>
<tr>
<th>Medium Information</th>
<th>Complete medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM (Gibco), 10% (v/v) FBS (Biochrom),</td>
<td></td>
</tr>
<tr>
<td>10 ng/mL basic fibroblast growth factor</td>
<td></td>
</tr>
<tr>
<td>(Peprotech), 2 mM L-glutamine, 1% B-27</td>
<td></td>
</tr>
<tr>
<td>(m/v) (Gibco), and 10^4 IU/mL penicillin/</td>
<td></td>
</tr>
<tr>
<td>streptomycin</td>
<td></td>
</tr>
<tr>
<td>LDMEM, 10% FBS (v/v), 1% streptomycin,</td>
<td></td>
</tr>
<tr>
<td>0.5 mmol/l 3-isobutyl-1-methylxanthine</td>
<td></td>
</tr>
<tr>
<td>(IBMX), 10 mg/L INS, 1.0 μM/L dexamethasone,</td>
<td></td>
</tr>
<tr>
<td>200 μM/L indomethacin</td>
<td></td>
</tr>
<tr>
<td>LDMEM, 10% FBS (v/v), 100 mM dexamethasone,</td>
<td></td>
</tr>
<tr>
<td>250 μM L-ascorbic acid-2-phosphate and 10 mM b-glycerophosphate</td>
<td></td>
</tr>
<tr>
<td>LDMEM, 10% FBS (v/v), 1% streptomycin,</td>
<td></td>
</tr>
<tr>
<td>medium which contained LDMEM medium, 5% FBS (v/v), 20 ng/mL FGF-4, 20 ng/mL HGF, 40 nmol/mL dexamethasone and ITS</td>
<td></td>
</tr>
<tr>
<td>LDMEM, 2% B27, 1% glutamine, 40 ng/mL bFGF, 20 ng/mL EGF</td>
<td></td>
</tr>
<tr>
<td>LDMEM, 2% B27, 1% glutamine, 40 ng/mL bFGF, 20 ng/mL EGF, 1% Nog, 50 μg/mL Vc, 10 ng/mL GDNF</td>
<td></td>
</tr>
</tbody>
</table>

Adipogenic differentiation and confirmation

Adipose-derived stem cells from passage 16 were plated onto 60-mm dishes. When the cells confluenced to 70%, the medium was changed to adipocyte-inducing medium. Cells from the control group were fed with DMEM medium. After 10 d, the induced cells were fixed with 4% (m/v) paraformaldehyde and stained with Oil-Red O for 30 min to visualize lipid droplets. The adipogenic specific genes LPL and PPARγ were detected by RT-PCR.

Osteogenic differentiation and confirmation

The ADSCs from passage 18 were plated onto 60-mm dishes for osteogenic differentiation. The cells were incubated with osteogenic-inducing medium when they confluenced to 80%. Cells from the control group were fed with DMEM medium. The cells were fixed with 4% paraformaldehyde (m/v) at 10 and 22 d after induction. These cells were also stained with alizarin to visualize the mineralized matrix. The osteogenic-specific genes OPN, COL1, RUNX2 were detected by RT-PCR.

Hepatic differentiation and confirmation

To induce hepatogenic differentiation of ADSCs, passage 13 cells were incubated on a 60-mm dish. When the cells confluenced to 50%, the medium was changed to hepatic-inducing medium. After 15 d, the cells were fixed with 4% (m/v) paraformaldehyde and stained with periodic acid Schiff to visualize the glycogen deposits. The hepatogenic specific genes AFP and ALB were detected by RT-PCR.

Epithelial cell differentiation and confirmation

The ADSCs from passage 8 were incubated on a 6-well plate. After the cells confluenced to 50%, the medium was changed to L-DMEM medium containing 10% FBS (v/v), 1% B27, 1% glutamine, 1% streptomycin, 10 ng/mL bFGF, and 15 ng/mL EGF. After 10 d, the
cells were confirmed with immunofluorescent assays of epithelial markers PCNA and CK18. The epithelial specific genes CK18 and E-Carderin were detected by RT-PCR.

**Neurogenic differentiation of ADSCs and confirmation**

The ADSCs from passage 9 were plated onto a 6-well plate. After the cells confluenced to 70%, the medium was changed to a neurogenic-induction medium. The induction was divided into 2 steps: i) the cells were incubated with the neurogenic induction medium-1 for 7 d; ii) the cells were then incubated with the neurogenic induction medium-2 for 7 d. The cells were confirmed with immunofluorescent assays of neural markers MAP2, GFAP, and β-tubulin. The neural-specific genes NF and map2 were detected by RT-PCR.

**Results**

**Isolation, culture, and morphological observation**

Primary cells isolated from adipose tissue adhered to plates and began to elongate after 48 h (Figure 1A-a). After about 3 d, the cells exhibited a fibroblast-like morphology (Figure 1A-b). Cells expanded rapidly and confluened to 90% 10 d later (Figure 1A-c). Cells were cultured up to passage 28 with most cells showing signs of senescence, such as vacuolization and karyopyknosis (Figure 1A-d).

**Identification of the ADSCs**

Specific surface antigen markers of ADSCs were detected by immunofluorescence and RT-PCR. Results of immunofluorescence
Figure 5. Multi-potential of adipose-derived stem cells. (a) After incubation in adipogenic for 15 days, the cells stained with oil-red (1,2) and 3 was for control. (b) RT-PCR showed the expression of adipogenic specific genes, such as LPL and PPAR-γ. 1 for the inducted group, 2 for the control group. (c) After incubation in osteogenic for 22 days, the cells stained with alizarin red (1,2) and 3 was for control. (d) RT-PCR showed the expression of osteoblast specific genes, such as osteopontin (OPN), collagen type 1 (COL1) and RUNX2. 1 for the inducted group, 2 for the control group. (e) After
incubation in hepatogenic for 15 days, the cells stained with periodic Acid-Schiff stain (1,2) and 3 was for control. (f) RT-PCR showed the expression of hepatogenic specific genes, such as AFB and ALP. 1 for the induced group, 2 for the control group. (g) The morphology of the induced ADSCs and undifferentiated ADSCS. (h) RT-PCR showed the expression of epithelial cells specific genes include CJ18 and E-Caderin. 1. induced group; 2. control group. (i) Immunofluorescence assay of the induced ADSCs. 1 — Phase contrast; 2 — DAPI; 3 — CK18; 4 — Merged.
staining demonstrated that the ADSCs were CD29, CD44, CD105, and Vimentin positive (Figure 2). The RT-PCR indicated that the ADSCs expressed CD29, CD71, CD73, CD105, and CD166 but didn’t express endothelial marker CD31 (Figure 1C).

**Growth kinetics**

Growth and proliferation of ADSCs were similar at P4, P14, P20, and P26 according to the growth curves analysis (Figure 3). After a latency phase of 1 to 2 d, cell growth entered the logarithmic phase, and reached the plateau phase at approximately day 8. The PDT was 28, 31, 32, and 36 h for P4, P14, P20, and P26, respectively.

**Colony-forming cell assay**

Colony formation was observed by microscopy after 6 d. Colony-forming levels were $37 \pm 0.2\%$, $30.6 \pm 0.1\%$, and $24 \pm 0.3\%$ for passages 4, 12, and 27, respectively, demonstrating the capacity of cultured ADSCs for self-renewal (Figure 4).

**Karyotype analysis**

The diploid chromosome number of pig ADSCs was $2n = 38$, consisting a pair of sex chromosomes per gender. There was no normal diploid chromosome ploidy missing or broken (Figure 5).

**Adipogenic differentiation of the ADPCs**

The adipogenic potential was evaluated by differentiation of post-confluent ADSCs. The obvious lipid droplet appeared after 10 d of induction, and it was in the cytoplasm. The lipid droplet was stained strongly with Oil O Red through fatty acids, and presented as bright red inclusions (Figure 5a). The expression of adipocyte specific markers LPL and PPARɣ was tested by RT-PCR for 10-day differentiated cells. The undifferentiated cells were tested as controls. The results are shown in Figure 5b.

**Osteogenic differentiation of the ADPCs**

Morphological changes were used as evidence for osteogenic differentiation during the induction. At the end of the 22-day induction period, calcium crystals appeared in the cytoplasm and the differentiation was confirmed by alizarin staining for calcium (Figure 5c). The expression of osteoblast specific markers OPN, COL1, and RUNX2 was tested by RT-PCR for the 10-day and 22-day differentiated cells. The undifferentiated cells were tested as controls. The results are shown in Figure 5d.

**Hepatic differentiation of the ADSCs**

Morphological changes in cultured ADSCs at passage 13 were observed. Along with the differentiation, the cells at passage 13 lost their fibroblastic morphology gradually and became a flatter and broader in shape; at the 15th day of induction, these cells formed a polygonal shape. The ability of glycogen-storage at differentiated cells was formed using PAS staining (Figure 5e) AFB, a mature functional hepatocyte-specific marker and AFP, a marker of immature hepatocytes, were tested by RT-PCR. Both differentiated cells expressed the AFB and ALP, but the undifferentiated cells were not expressed (Figure 5f).

**Epithelial cells differentiation of the ADSCs**

During the induction, many morphological changes appeared in the cultured cells. The cells at passage 6 lost their fibroblastic morphology gradually and became flatter and broader in shape; at the 13th day of differentiation, the cells appeared cobblestone in shape. Induction of 13-day cells was further confirmed by immunofluorescence stain for CK18 (Figure 5g). CK18 is a specific antibody for epithelial cells. Specific surface markers were evaluated by RT-PCR and the undifferentiated cells were used as controls (Figure 5i).

**Neurogenic differentiation of ADSCs**

The ADSCs were pre-induced for 7 d, after which spindle-shaped cells began to contract with an irregular form. Following induction, cell bodies further contracted and became round, triangular, or cone-shaped with multipolar processes. The processes continued to grow with many branches forming and cone-like terminal expansions were observed. A number of cells demonstrated very long processes, which appeared similar to the long axon of neurons. Immunofluorescence staining results showed that the MAP2, GFAP, and β-tubulin markers of neural cells were expressed in the differentiated cells (Figure 5j). The RT-PCR showed that the induced group cells were positive for NF and map2.

**Discussion**

In this study, ADSCs were successfully isolated from the visceral omental region of pigs. The tissues were washed with PBS 8 times and digested through 1% I type collagenase for 1 h. The cells were cultured in a medium that contained 10% FBS, 1% B27, 1% glutamine, 1% streptomycin, and 10 ng/mL bFGF. Because bFGF can promote mitosis and stimulate anabolism, it is important to the growth of ADSCs and B27 is a secondary growth factor that provides nutrients for ADSCs. The medium can guarantee ADSCs in the best state of growth. There were few cells that had adhered 48 h after inoculation. The cells first confluence to 80% was 10 d after inoculation. During the first 10 d, the medium was changed every 2 d. Later, cells were passaged stably over 2 d on average. At the 26th passage of ADSCs, the characteristics of aging appeared and the times of passage were prolonged by 4 d. This was maybe due to the cells themselves, several times the enzyme digestion, or the medium. Further research is required to discover the specific reasons for this.

There were no specific surface markers used to identify and screen for MSCs. So RT-PCR, immunofluorescent staining, and flow cytometry were used to test for the surface markers of ADSCs. The results showed ADSCs highly expressed CD29, CD44, CD71, CD73, CD90, CD166, and vimentin, weekly expressed CD105, and were unexpressed CD31.

We explored the ability of clone formation for ADSCs at passages 4, 12, and 26. Results showed that ADSCs have a strong ability of clone formation in a good growth state. In order to detect whether there was any variation in the proliferation of ADSCs, we conducted karyotype analysis. Results demonstrated there was no chromosome missing or broken in the ADSCs.
In order to study the pluripotency of ADSCs, we differentiated the cells into ectoderm, mesoderm, and endoderm. We differentiated the ADSCs into epithelial and neurogenic cells for ectoderm. The results of cell differentiation were verified by cell morphology, RT-PCR, and immunofluorescence stain. For epithelial cell morphology, the control groups were typical fibroblast-like shape and the induced group cells changed into a cobblestone-like shape. For RT-PCR, the control groups didn’t express epithelial-cell specific markers CK18 and E-cadherin, while the induced group cells highly expressed CK18 and E-cadherin. For immunofluorescence staining, the induced cells were positive to the antibody CK18, which is a marker for epithelial cells. For the neurogenic cells, the control group cells still retained a fibroblast-like shape, while the induced group cells changed into a typical neuronlike morphology and long axons were observed. Results of immunofluorescence staining showed the induced group cells were positive for GFAP, MAP2, and β-tubulin. The cells were then differentiated into adipocytes and osteoblasts for mesoderm. And we detected the results by cell morphology, staining and RT-PCR. For adipogenic differentiation, the induced cells appeared as obvious fat drops in the cytoplasm, expressed the adipocytes specific markers LPL and PPARy, and the fat drops stained red with Oil-Red O. For osteogenic differentiation, the induced cells had an oval-like shape with many aggregations. Along with the increase of aggregations, calcium nodules were emerging in the cytoplasm. The induced cells were stained with alizarin, and with Oil-Red O and expressed the osteoblast specific markers OPN, COL1, and RUNX2. For hepatic differentiation, the induced cells changed from a fibroblast-like shape to a polygonal or round shape. And RT-PCR results showed the induced cells expressed mRNA for ALB and AFP, while these were not expressed in control groups. We evaluated the efficiency of differentiation by PAS staining and the induced cells were dyed purple in the cytoplasm. Results showed that ADSCs have the ability of crossing layer differentiation. Though ADSCs can differentiate into 3 layers, the function of induced cells was not tested; therefore, more research is needed in this capacity.

Adipose tissue is an abundant source of mesenchymal stem cells, which have shown promise in the field of regenerative medicine. Various clinical trials have shown the regenerative capability of adipose-derived stem cells in subspecialties of medical fields such as plastic, orthopedic, oral maxillofacial, and cardiac surgeries. Breast reconstruction and augmentation trials have been reported by Yoshimura et al (11–12). Adipose-derived stem cells were first used to stimulate craniofacial bone repair in calvarial defects (13). These stem cells have been used to heal chronic fistulas in Crohn’s disease (14–15).

Myocardial infarction is a life-threatening medical emergency, which is the greatest cause of death in developing countries. Until now, myocardial infarction is mainly dealt with through drugs and surgery, which can have many side effects on patients. Adipose tissue taken from around the heart has shown that ADSCs can differentiate into myocardial cells in vitro. Stem cell therapy is an effective way to cure many diseases, so it is significant if ADSCs derived from visceral (omental region) could be differentiated into myocardial cells and be used to induce cells to cure myocardial infarction.

In conclusion, ADSCs were isolated from visceral omental region of the pig, and the self-renewal ability and differential potential were evaluated in vitro. The present study illustrates the potential application of adipose tissue as an adult stem cell source for regenerative therapies.

References

Clinical staging in bitches with mammary tumors: Influence of type and histological grade
Lígia F. Gundim, Camila P. de Araújo, William T. Blanca, Ednaldo C. Guimarães, Alessandra A. Medeiros

Abstract
Breast tumors are the most common tumors in dogs and the study of disease prognostic factors is important for establishing the appropriate treatment protocols. The purpose of this study was to clinically stage mammary tumors of bitches and correlate the stages with histological type and grade. The tumors of 63 dogs were clinically staged based on the findings of tumor sizing, lymph node evaluation, and radiographic examination. After surgical excision, the tumors were classified histologically and graded. The relationship between the tumor grade, stage, and histological type was evaluated using a binomial test. Stage I tumors were the most numerous (31.75%), followed by tumors at stages II, III, IV, and V. Animals with histological grade I carcinomas presented stage I, II, or III tumors more frequently and stage IV and V tumors less frequently. The number of animals with simple carcinomas that were at stage I of the disease was greater than that at stage V. Carcinomas in the mixed tumors were less aggressive; however, the small number of animals in stage V of the disease made any statistical association impossible. The complex carcinomas presented with the invasion of the lymph nodes and less cellular differentiation in a larger number of animals than did simple carcinomas. Histological grading proved to be the best parameter for the prognostic evaluation of the breast carcinomas.

Résumé
Les tumeurs mammaires sont les tumeurs les plus fréquentes chez les chiens et l'étude des facteurs de pronostic de la maladie est importante afin d'établir les protocoles de traitement appropriés. Le but de cette étude était de déterminer le stade clinique des tumeurs mammaires de chiennes et de corrélérer les stades au type histologique et le grade. Les tumeurs de 63 chiens ont été classées cliniquement en se basant sur la taille de la tumeur, l'évaluation des nœuds lymphatiques, et l'examen radiographique. Après excision chirurgicale, les tumeurs ont été classées histologiquement et un grade attribué. La relation entre le grade de la tumeur, le stade, et le type histologique a été évaluée en utilisant un test binomial. Les tumeurs de Stade I étaient les plus nombreuses (31,75 %), suivies des tumeurs des stades II, III, IV, et V. Les animaux avec un carcinome de grade I histologiquement présentaient des tumeurs de stade I, II, ou III plus fréquemment et des tumeurs de stade IV et V moins fréquemment. Le nombre d'animaux avec un carcinome simple qui était au stade I de la maladie était plus grand que ceux au stade V. Les carcinomes dans les tumeurs mixtes étaient moins agressifs; toutefois, le petit nombre d'animaux au stade V de la maladie rendait impossible toute association statistique. Les carcinomes complexes se présentaient avec une invasion des nœuds lymphatiques et moins de différenciation cellulaire dans un plus grand nombre d'animaux que les carcinomes simples. Le pointage histologique s'est avéré être le meilleur paramètre pour l'évaluation du pronostic des carcinomes mammaires.

Introduction
Breast tumors are the most common tumor in female dogs (1,2). They have no breed predisposition and mainly affect senile animals and those not castrated before the first heat (1). Dogs may be used as the natural surrogate model for the evaluation of tumors in the human female breast because of similarities between the clinical, pathological, epidemiological, and developmental characteristics of tumors in dogs and humans (3).

Several systems have been proposed to assess the prognosis of these animals. One such system is the TNM, where: T — describes the tumor size; N — lymph node metastasis; and M — distant metastasis (4). There are studies showing a strong correlation between the tumor size and malignancy (5) with the presence of the metastases in the lymph nodes and the survival of the affected animal (6,7). In human female patients, the TNM system is still widely used as a predictive factor for mammary carcinomas, although more modern methods such as biomarkers are available (8).

Another method that can be used for the prognostic evaluation of tumors is histological grading (9). Grade III tumors have been associated with a low survival time (10,11). It has also been established, with regard to the histological classification of the tumors,
that complex carcinomas exhibit less aggressive behavior than the simple carcinomas. Of the simple carcinomas, anaplastic carcinoma is the most aggressive, followed by solid papillary, and tubule carcinomas (1).

Owing to the paucity of studies in dogs assessing histological gradation, histological typing, and staging as prognostic factors, this study aimed to examine the clinical staging of breast malignancies in bitches and correlate the stages of tumors with their histological type and grading.

### Materials and methods

#### Sampling

The study included 63 dogs with mammary tumors that visited the Veterinary Hospital of the Federal University of Uberlândia voluntarily. Inclusion criteria for the selection of animals were: bitches with malignant mammary neoplasia, no pre-existing diseases, availability of documented medical history, and previous histopathological and radiological examinations. All dogs included in the study were intact (not spayed).

#### Clinical staging

In order to determine the clinical stage of the tumors, chest X-rays were performed at the ventrodorsal and right and left laterolateral positions. The animals also underwent a complete physical examination, including the careful palpation of the mammary glands and verification of the general clinical condition of the animal. During the clinical examination, both the mammary chains and the regional lymph nodes were explored by examining the number of affected mammary glands, the presentation of tumor masses, the tumor size, and the surface of the lymph nodes involved.

All dogs underwent surgical removal of breast and regional lymph nodes. Fragments of these tissues were collected and subjected to histopathological evaluation. Samples were fixed in 10% buffered formalin and subjected to histological processing (12), followed by the preparation of the histopathological slides stained with hematoxylin-eosin (H&E) stain.

Using data on tumor sizes, radiological findings, and assessment of the presence of the metastatic lymph nodes by histopathology, the tumors of the bitches were staged according to the TNM system (Table I) (4).

### Histological classification

Breast tumors were histologically classified according the method of Cassali et al (2). Neoplasms were grouped according to their histogenesis into complex carcinomas, simple carcinomas (tubular, papillary, solid, and anaplastic carcinomas), and mixed tumors. The invasive lobular carcinomas, inflammatory carcinomas, carcinomas, carcinomas in situ, osteosarcomas, and fibrosarcomas were classified as other types of tumors.

#### Histologic grading

Histologic grading of breast tumors was performed by 2 observers according to the method of Elston and Ellis (9), in which the tumors were classified as being grade I, II, or III, on the basis of the formation of the tubules, nuclear pleomorphism, and mitosis number.

#### Statistical analysis

The relationship between the histological grade of tumors, their clinical staging, and their histological type, was evaluated using the binomial test for comparing 2 proportions, with a $P = 0.05$ significance value. The statistical analyses were performed using the Action program, version 2.9 (Software Action. Estatcamp-Consultoria em estatística e qualidade, São Carlos — SP, Brasil.)

### Results

Animals aged 8 to 14 years were the most affected (77.19%). Mongrel dogs showed the highest prevalence of breast tumors (44.26%), followed by poodles with 19.67%. No dogs were castrated prior to the removal of breast tumors, although some were castrated at the time of tumor resection. The distribution of bitches according to the results of the clinical staging is shown in Table I.

The most frequent tumor type was tubular carcinoma, followed by carcinoma in mixed tumors (Table III). Among the animals...
with simple carcinomas, most were classified as stage I, with only 3 animals presenting lung metastases (stage V) and 10 animals exhibiting lymph node metastases (stage IV). It was observed that dogs with simple carcinomas were most frequently classified as stage I and less frequently as stage V ($P = 0.026$). None of the animals in the mixed tumor group were classified as stage V and only one was classified as stage IV. However, there were no significant differences in the frequency of bitches among the clinical stages for this histological type ($P = 0.07$) (Table IV).

With regard to the histologic grading of the tumors, 60 carcinoma samples were graded; tumors diagnosed as osteosarcomas and fibrosarcomas were excluded since carcinomas only were evaluated for histological grading. Among the tumors classified as simple carcinomas, 40% (14/35) were classified as grade I, 50% (18/36) as grade II, and 11.1% (4/36) as grade III. Among the complex carcinomas, 85.7% (6/7) were classified as grade I and 14.28% (1/7) as grade II. Among the carcinomas in the mixed tumor group, 68.8% (11/16) were classified as grade I and 31.3% (5/16) as grade II. It was observed that the animals with the histological grade I presented stage I, II, or III tumors more frequently, and stage IV ($P = 0.01$) and stage V ($P = 0.0009$) tumors less frequently (Table V).

### Discussion

Previous studies have reported tumor metastases in less than 50% of dogs, which corresponds with the findings of this study. A retrospective study of 99 dogs in the United States reported 25% of the animals at stage I, 12% at stage II, and 15% at stage III of the disease (13). However, a study in Brazil that examined 36 dogs described 22.2% of the animals as stage I and 75% as stage II of the disease; none of the animals were classified as stage III (14).

Given that the differentiation between stages I, II, and III is based only on the tumor size, another study conducted in Brazil reported that 35% of malignant tumors were smaller than 5.0 cm (11), while other studies have reported that neoplasms measured less than 3.0 cm [48.81% (15) and 44.7% (16), respectively].

A study involving human female subjects reported that 55.3% of patients presented tumors smaller than 3 cm and that the tumor diameter negatively affected the chance of survival despite resection (7). The smaller tumor size in these female subjects might have been due to early diagnosis. A similar correlation was observed herein which might be due, in part, to the owners seeking medical attention for swelling in the breasts sooner than usual.

With regard to the bitches diagnosed with the lymph node invasion in the present study (29.7%), previous studies conducted in Portugal and Colombia showed similar frequencies of occurrence [298% (16) and 30% (17), respectively]. Another study, however, had classified only 15.46% of the animals as stage IV (13).

The frequency of distant metastases in the present study was 7.9%. Previous studies have also reported similarly low frequencies of bitches at this stage of the disease; Ribas et al (18) and Gomes et al (17) reported that 8.3% and 16.7% of dogs were diagnosed with pulmonary metastasis, respectively. However, a negative radiological finding does not prove the absence of metastases, since the detection of tumors is challenging owing to their smaller sizes (19). Moreover, highly accurate diagnostic methods such as magnetic resonance imaging (MRI) and positron emission tomography (PET) scans are rarely practiced in veterinary medicine. Another reason for the fewer number of dogs at stage V of the disease is the fact that these animals are often already at advanced stages of the disease without having received any surgical treatment.

With regard to the frequency of tumors according to histological type, previous studies have reported that tubular carcinoma is the most common (20,21), which corresponds to the findings in this study. However, the incidences of other types of tumors reported by different studies vary. The different histological classification systems used for the canine mammary tumors makes it difficult to compare the results of the various studies. The solid carcinoma has been reported as the second most frequent type of carcinoma in a previous study (20); however, another study reported the complex carcinoma as being the second most frequent (21). Carcinoma in mixed tumors also appears as one of the more frequent types of carcinomas (22), and in the present study, it was found to be the third most frequent.

The simple carcinomas were found to have the highest frequency of occurrence in this study when the tumors were grouped on the basis of their histological origin. The simple carcinomas have also been reported as the most frequent type of tumors in studies conducted in Japan (23), Brazil (24), and Iran (21).

Several authors have reported that simple carcinomas are more aggressive and have a worse prognosis than complex carcinomas (25,26). Therefore, the clinical staging of simple carcinomas in the present study was unexpected and can be attributed to the fewer number of the animals at the stages II, III, and V.

However, the staging of the carcinomas in mixed tumors in this study revealed the lower aggressiveness of these tumors, as has previously been described by Cassali et al (26), who also reported that these tumors have a better prognosis compared to other carcinomas. However, in the present study, the statistical corroboration of this fact was hindered because none of the animals with carcinomas in mixed tumors were at stage V.

### Table IV. Frequency of histological types of breast carcinomas according to the clinical stage

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Stage V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple carcinoma</td>
<td>13</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Complex carcinoma</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Carcinoma in mixed tumor</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

### Table V. Frequency of histological grade of breast carcinomas according to the clinical stage

<table>
<thead>
<tr>
<th>Grade</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Stage V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>11</td>
<td>6</td>
<td>5</td>
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<td>2</td>
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<td>3</td>
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<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
With regard to the complex carcinomas, we did not expect a large number of the animals to present lymph node invasions (stage IV) since Rasotto et al (27) had previously shown that 66.6% of the simple carcinomas in their study had metastasized to the lymph nodes and in 81.01% of the cases, invasion of the lymphatic vessels was observed; whereas, among the complex carcinomas, only 11.11% had metastasized to the lymph nodes and 20% showed lymphatic invasion.

The time-evolution of the tumor might affect the occurrence of the metastases. The larger tumors usually have a longer development duration, which increases the likelihood of metastasis (28). Unfortunately, the determination of the duration of tumor progression depends on the owner’s observation of the condition and is therefore often unclear.

In the histological grading findings, complex carcinomas showed less differentiation compared to simple carcinomas, as has been reported previously (27,10). The degree of differentiation of simple carcinomas is a matter of debate; it has been reported that this histological type of carcinoma has no association with the degree of differentiation (27) and that their classification as grade III (10) is frequent. The discrepancies between the results of the various studies might be because of the subjectivity of the gradation method, which can vary according to the reviewer. Another hypothesis is that a greater number of tubular tumors were classified as carcinomas in this study since tubule formation is one of the parameters considered for gradation, and tubular carcinomas tend to attain lower scores in the evaluation.

Histological grade is a factor that can influence the clinical staging of a tumor. A study in Slovenia reported that 64.71% of animals had stage I and grade I tumors, a result that is comparable to what we presented in this study (29). Similarly, a study conducted in Madrid described a significant association between the histological grade and the clinical staging of tumors (30). Other previous studies have also correlated the type and histological grade of the tumors with the overall survival rate (16).

A limitation of the present study was the fewer number of animals at stage V of the disease. Although mixed tumor carcinomas typically show less aggression, fewer metastases, and lower cell differentiation, whereas simple carcinomas are more aggressive, this association could not be proven statistically because of the fewer number of animals at stage V. It can, however, be concluded that the histological grading proved to be the best parameter for the prognostic evaluation of breast carcinomas.

References

20. Salas Y, Márquez A, Díaz D, Romero L. Epidemiological study of mammary tumors in female dogs diagnosed during the


Tramadol does not enhance sedation induced by acepromazine in dogs
Eduardo R. Monteiro, Renan B. Lobo, Juarez S. Nunes Jr, Julia P.P. Rangel, Flavia S. Bitti

Abstract
The sedative effect of acepromazine combined with 2 doses of tramadol [3 and 5 mg/kg body weight (BW)] was compared with the sedative effect of acepromazine alone in dogs and the effects of each sedative protocol on cardiorespiratory variables were examined. This was a prospective, randomized, blinded, crossover study. Each of 6 dogs received 3 treatments at 1-week intervals. During all anesthetic episodes, dogs received 0.05 mg/kg BW acepromazine. Approximately 25 min later, dogs were given physiological saline (control) or tramadol [3 mg/kg BW (TR3) or 5 mg/kg BW (TR5)]. All drugs were administered intravenously. Variables evaluated included heart rate (HR), respiratory rate (RR), systolic, mean, and diastolic blood pressures (SAP, MAP, and DAP), and sedation [by use of a simple descriptive scale (SDS, range: 0 to 3) and a numeric rating scale (NRS, range: 0 to 10)]. Variables were recorded 25 min after acepromazine and for 80 min after saline or tramadol. Acepromazine administration resulted in mild sedation in most dogs and decreased RR, SAP, MAP, and DAP in all treatments. Tramadol administration did not significantly increase SDS or NRS scores compared to acepromazine alone. The only exception to this rule was observed at 20 min after TR3, when NRS was higher in this group than in the control treatment. Administration of tramadol (TR3 and TR5) decreased HR. Under the conditions of this study, sedation induced by acepromazine with tramadol was similar to that of acepromazine alone. The main adverse effects of the combination were a decrease in blood pressure and HR, without clinical significance.

Résumé
L’effet sédatif de l’acépromazine combiné à deux doses de tramadol [3 et 5 mg/kg de poids corporel (PC)] a été comparé à l’effet sédatif de l’acépromazine seul chez des chiens et les effets de chaque protocole de sédation sur des variables cardio-respiratoires ont été examinés. Il s’agissait d’une étude prospective croisée, randomisée, réalisée à l’aveugle. Chacun des six chiens a reçu trois traitements à des intervalles de 1 semaine. Durant tous les épisodes anesthésiques, les chiens ont reçu 0,05 mg/kg PC d’acépromazine. Environ 25 min plus tard, les chiens ont reçu de la saline physiologique (témoin) ou du tramadol [3 mg/kg PC (TR3) ou 5 mg/kg PC (TR5)]. Toutes les drogues étaient administrées par voie intraveineuse. Les variables évaluées incluaient le rythme cardiaque (RC), le rythme respiratoire (RR), les pressions sanguines systolique, moyenne, et diastolique (PSS, PSM, et PSD), et la sédation [en utilisant une échelle descriptive simple (EDS, écart : 0 à 3) et une échelle de gradation numérique (EGN, écart : 0 à 10)]. Les variables ont été enregistrées 25 min après l’acépromazine et pendant 80 min après l’administration de saline ou de tramadol. L’administration d’acépromazine a résulté en une légère sédation chez la plupart des chiens et on nota une diminution de RR, PSS, PSM, et PSD avec tous les traitements. L’administration de tramadol ne fit pas augmenter de manière significative les pointages EDS et EGN lorsque comparée à l’acépromazine seul. La seule exception à cette règle a été observée à 20 min après TR3, alors que l’EGN était plus élevée dans ce groupe comparativement au témoin. L’administration de tramadol (TR3 et TR5) entraîna une diminution du RC. Dans les conditions de la présente étude, la sédation induite par l’acépromazine avec du tramadol était similaire à celle de l’acépromazine seul. Les principaux effets adverses de la combinaison étaient une diminution de la pression sanguine et du RC, mais sans signification clinique.

Introduction
Acepromazine is the phenothiazine derivative most commonly used for sedation in dogs. The drug has been found to possess antagonistic action at dopaminergic receptors within the brain; this may explain the mechanism by which acepromazine induces sedation (1). Based on extensive data on the use of acepromazine in dogs, the degree of sedation following acepromazine administration in canine patients is mild to moderate (2–4).

It has been reported that acepromazine is devoid of analgesic properties (1). Therefore, acepromazine is commonly administered in combination with opioid analgesics to provide sedation and analgesia, which facilitates handling of dogs for placement of venous catheters, preparation for surgery, and diagnostic procedures. In addition to providing analgesia, there is evidence that an opioid analgesic enhances the degree of sedation induced by acepromazine in dogs. In a previous study, methadone, morphine, butorphanol, and tramadol were compared and methadone appeared to be the most effective for this purpose (3).

Tramadol has been classified as an opioid analgesic although it was found to provide analgesia by opioid and non-opioid mechanisms (5). In humans, its opioid analgesic properties were associated with clinical effects of the drug, such as respiratory depression and hypotension, which are not observed in dogs. The purpose of this study was to determine if tramadol increases sedation induced by acepromazine in dogs, and whether the combination of tramadol and acepromazine is superior to either agent alone.
with the production of the active metabolite O-desmethyltramadol (M1) (6), which was found to possess greater affinity for opiate receptors than the parent drug tramadol (7). Drowsiness has been reported as an adverse effect after tramadol administration in humans (8).

There is conflicting evidence about the sedative effect of tramadol in dogs. In one study, a dose-related sedative effect was observed in dogs administered 1, 2, or 4 mg/kg body weight (BW) tramadol by intravenous (IV) injection (9), whereas no sedation was evidenced after IV tramadol (approximately 4 mg/kg BW) to dogs in another study (10). When combined with acepromazine, tramadol appeared to be less effective in enhancing the degree of sedation induced by the phenothiazine than methadone, morphine, and butorphanol (3). However, the dose of tramadol (2 mg/kg BW) was not equipotent to the dose of other opioids and this may have accounted for the low efficacy of tramadol in this later study (3). The present study aimed to compare the sedative effect of acepromazine combined with 2 different doses of tramadol [3 mg/kg BW (TR3) or 5 mg/kg BW (TR5)] to the sedative effect of acepromazine alone in dogs. We hypothesized that tramadol would enhance sedation induced by acepromazine. A second objective of this study was to evaluate the effects of each sedative protocol on cardiorespiratory variables of dogs.

### Table I. Medians (interquartile range) simple descriptive scale (SDS, range: 0 to 3) and numeric rating scale (NRS, range: 0 to 10) sedation scores in 6 dogs. Sedation was assessed at 25 min after administration of acepromazine, 0.05 mg/kg body weight (BW), IV (time point ACP) and at 20, 40, 60, and 80 min after administration of physiological saline (control) IV, 3 mg/kg BW tramadol (TR3) or 5 mg/kg BW tramadol (TR5) IV

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<td>(1.0 to 1.3)</td>
</tr>
<tr>
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</tr>
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<tr>
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<td>(3.8 to 6.3)</td>
<td>(2.5 to 5.3)</td>
<td>(1.8 to 5.0)</td>
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<tr>
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<td>3.0</td>
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<td>(3.8 to 5.3)</td>
<td>(2.0 to 4.0)</td>
<td>(1.8 to 4.0)</td>
</tr>
</tbody>
</table>

\( a \) Significant difference compared to control treatment (P < 0.05).  
\( b \) Significant difference compared to time point ACP.

was confirmed by physical examination and laboratory evaluation [complete blood (cell) count (CBC) and biochemistry profile].

This was a prospective, randomized, blinded, crossover study. Each dog received 3 treatments on different occasions, with 1-week washout intervals. During all anesthetic episodes, dogs were administered acepromazine (Acepran 0.2%; Vetnil, Louveira, São Paulo, Brazil), 0.05 mg/kg BW IV. Approximately 25 min after the administration of acepromazine, physiological saline (control) IV or tramadol IV (Cloridrato de tramadol 50 mg/mL; Hipolabor Farmacêutica, Belo Horizonte, Minas Gerais, Brazil) at TR3 or TR5, was given.

Dogs were fasted for 12 h prior to each experiment, but had free access to water. A 20-gauge catheter was introduced into a cephalic vein and the dogs were acclimated for 20 min to the laboratory environment. Baseline values for each variable were then recorded with the dogs gently restrained in lateral recumbency. Heart rate (HR) was measured with a stethoscope and respiratory rate (RR) was counted by observing chest wall movements. An oscillometric device (PetMap Classic; Ramsey Medical, Tampa, Florida, USA) was used for indirect measurement of systolic, mean, and diastolic blood pressures (SAP, MAP, and DAP, respectively). The blood pressure cuff was positioned proximal to the carpus in the nondependent limb and the cuff size was chosen according to the manufacturer’s directions. For every time point, 5 consecutive measurements of blood pressure were performed and averaged.

Sedation was scored by use of a simple descriptive scale (SDS) and a numeric rating scale (NRS). The SDS ranged from 0 to 3 as follows: 0 — no sedation; 1 — mild sedation, less alert but still active; 2 — moderate sedation, drowsy, recumbent but can walk; or 3 — intense sedation, very drowsy, unable to walk (11,12). The NRS ranged from 0 to 10 with the production of the active metabolite O-desmethyltramadol (M1) (6), which was found to possess greater affinity for opiate receptors than the parent drug tramadol (7). Drowsiness has been reported as an adverse effect after tramadol administration in humans (8).

### Materials and methods

The present study was initiated after approval by the institutional Animal Research Ethical Committee (protocol 324-2014). Six healthy adult crossbreed dogs (4 male and 2 female) were used. Average weight of the dogs was 18.7 ± 3.1 kg (mean ± SD). Healthy status
0 to 10, where 0 represented no sedation and 10 represented the most sedation possible (12). For the NRS, only whole numbers could be selected. A single observer, unaware of the treatment administered, was responsible for scoring SDS and NRS values. This person was familiar with both scoring systems. Dogs were initially observed without interaction with the observer. Thereafter, objective variables (HR, RR, SAP, MAP, and DAP) were recorded. Finally, the observer encouraged the dogs to stand and walk. Based on noninteractive and interactive behaviors, the observer recorded SDS and NRS scores.

After recording baseline variables, the dogs were administered acepromazine through the cephalic catheter. Within 20 to 25 min after acepromazine administration, all variables were recorded (time point ACP). Thereafter, physiological saline (control), TR3, or TR5 was administered over 1 min through the cephalic catheter and all variables were reassessed at 20 min intervals for 80 min (time points 20, 40, 60, and 80).

Sample size calculation was performed using G*Power for Windows Version 3.1.6 (Heinrich Heine Universität Düsseldorf, Germany) and was based on sedation scores reported for dogs in previous studies (2–3). Results of the power analysis indicated 6 dogs would be necessary to detect 1.0 point differences between groups for SDS scores and 3.0 point differences between groups for NRS scores with a power of 80% at 5% level of significance.

Statistical analyses were performed by use of a computer software (Prism 5.0; GraphPad Software, La Jolla, California, USA). Data distribution was analyzed by the Kolmogorov-Smirnov test. For normally distributed variables (HR, RR, SAP, MAP, and DAP), comparisons between treatments were analyzed by a 2-way repeated measures analysis of variance (ANOVA) with time and treatment as factors. When a significant difference between treatments was identified, a Bonferroni correction for multiple comparisons was used to determine what treatments differed. A 1-way repeated measures
ANOVA was performed to detect differences over time within each treatment. If a significant difference was detected, a Dunnett’s test for multiple comparisons was used to compare all time points with time point ACP. For comparisons between treatments and over time in sedation scores, a Friedman test was performed and post hoc analysis was conducted by use of the Dunn’s test for multiple comparisons. Differences were considered significant if $P < 0.05$.

**Results**

All 6 dogs completed the study. Acepromazine administration resulted in mild sedation in most dogs (Table I, Figure 1). Heart rate did not change significantly after acepromazine but a decrease was observed in SAP, MAP, DAP, and RR in all treatments at time point ACP (Table II).

Administration of the experimental treatment (time points 20 to 80 min) did not significantly increase SDS or NRS scores compared to time point ACP. The only significant difference between treatments in sedation scores was observed at 20 min, when NRS was higher in the TR3 treatment compared to the control treatment. At 80 min, NRS scores decreased in all treatment groups compared to the values at time point ACP (Table I). The distribution of SDS scores in each treatment is summarized in Figure 1.

Blood pressure and RR did not differ among treatments throughout the study. In addition, SAP, MAP, DAP, and RR did not change significantly after administration of the experimental treatment. The only exception to this rule was observed at time point 40, when DAP was significantly higher in TR5 compared to the value at time point ACP. Administration of tramadol (TR3 and TR5) resulted in significant decreases in HR from 20 to 80 min and the value for the TR3 treatment was significantly lower compared to the control treatment at 40 min (Table II).

**Discussion**

The present study revealed that combining TR3 or TR5 with 0.05 mg/kg BW acepromazine failed to enhance the degree of sedation induced by acepromazine. Mild to moderate sedation was observed after administration of acepromazine alone or the combination acepromazine-tramadol.

On the basis of SDS scores, the findings of this study are in agreement with previous studies that, in dogs, sedation following acepromazine alone ranges from mild to moderate (3,13). After intramuscular administration of acepromazine in dogs, peak sedative effect appears to occur within 15 to 30 min (2,4). In the present study, SDS and NRS scores assessed from 20 to 80 min in the control

### Table II. Mean ± SD heart rate (HR), systolic, mean and diastolic blood pressures (SAP, MAP, and DAP), and respiratory rate (RR) in 6 dogs before administration of any drug (baseline, BL), at 25 min after administration of acepromazine (time point ACP), 0.05 mg/kg body weight (BW), IV and at 20, 40, 60, and 80 min after administration of physiological saline (control) IV, 3 mg/kg BW tramadol (TR3) or 5 mg/kg BW tramadol (TR5), IV

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<tr>
<th></th>
<th>BL</th>
<th>ACP</th>
<th>20</th>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>97 ± 12</td>
<td>93 ± 20</td>
<td>90 ± 21</td>
<td>86 ± 16</td>
<td>77 ± 11</td>
<td>81 ± 10</td>
</tr>
<tr>
<td>TR3</td>
<td>85 ± 10</td>
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<td>TR5</td>
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<td>73 ± 8</td>
</tr>
<tr>
<td>SAP (mmHg)</td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
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<tr>
<td>Control</td>
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<tr>
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</table>

* Significant difference compared to time point ACP.

b Significant difference compared to the control treatment ($P < 0.05$).
treatment were not significantly greater than at time point ACP (approximately 25 min after acepromazine administration). These results suggest that peak sedative effect after IV acepromazine occurs within 25 min after administration of the drug. If sedation was assessed more frequently in this study, it would be possible to determine more precisely the period of time between IV acepromazine administration and peak sedative effect.

When an opioid analgesic is administered in combination with acepromazine, sedation is expected to be greater than after acepromazine alone, but there is conflicting evidence. In one study, intramuscular acepromazine-methadone (0.05 mg/kg BW and 0.5 mg/kg BW, respectively) resulted in non-significantly increase in sedation score compared to 0.1 mg/kg BW acepromazine (2). In another study, sedation induced by acepromazine (0.05 mg/kg BW) was significantly improved when morphine (0.5 mg/kg BW), methadone (0.5 mg/kg BW) or butorphanol (0.2 mg/kg BW) were administered 15 min after acepromazine via IV injection (3). Conversely, the combination of acepromazine (0.05 mg/kg BW) with hydromorphone (0.1 mg/kg BW) failed to induce greater sedation than acepromazine alone (4). The discrepancies between studies are probably related to differences on the scoring systems used to subjectively assess sedation, routes of drug administration and pharmacological characteristics of opioid analgesics used.

In a previous study in dogs, sedation assessed after combining acepromazine (0.05 mg/kg BW) with tramadol (2 mg/kg BW) was only mildly increased and for a short period (15 min), compared to sedation assessed after acepromazine alone (5). In the present study, it was hypothesized that higher doses of tramadol (TR3 or TR5), combined with acepromazine, would result in consistently greater sedation scores than acepromazine alone. No significant differences were observed in SDS scores between the control treatment and the TR3 and TR5 treatments. In addition, no significant difference in NRS scores were detected between the control and TR5 treatment and NRS scores in the TR3 treatment were significantly higher than in the control treatment at a single time point (20 min) only. These findings indicate there is no clinically important enhancement in sedation induced by acepromazine when doses of tramadol as high as TR5 are combined with phenothiazine.

The inefficacy of tramadol in enhancing the degree of sedation induced by acepromazine in dogs may be related to its pharmacokinetic and pharmacodynamic characteristics. Sedation following opioid administration is attributable to agonistic action at opioid receptors at brain sites (5). In cloned human opioid receptors, a greater affinity for opioid receptors was found for the M1 metabolite than for the parent drug tramadol (7). It has been reported that dogs can only produce low levels of M1 and that this metabolite has a fast elimination phase in this species (9). Therefore, sedation may be impaired in dogs because of its inability to produce significant amounts of M1. This statement is supported by results of a previous study where administration of tramadol orally (11 mg/kg BW) or by IV (4.4 mg/kg BW), did not result in detectable signs of sedation, whereas administration of M1 resulted in mild sedation in dogs (10).

Acepromazine administration did not change HR in the present study. Heart rate measurements from 20 to 80 min in the TR3 and TR5 groups were typical of opioid analgesics in dogs. A sustained decrease of 25% to 30% in HR was observed in both groups compared with time point ACP. The negative chronotropic effect of opioid analgesics is related to their actions within the medulla oblongata increasing vagal tone (14). Although the effect of opioids on HR is dose-related, there is a plateau after which further increases in dose does not result in more bradycardia (15). In agreement with this previous study, our findings revealed the dose of tramadol did not influence the magnitude of the decrease in HR.

A decrease in SAP, MAP, and DAP was observed after acepromazine administration in all treatment groups. The effect of acepromazine on blood pressure is thought to result from antagonistic action at alpha-adrenergic receptors within vascular beds resulting in vasodilation (16). Moreover, in one study in dogs, acepromazine administration resulted in a decrease in stroke volume, which could also reduce blood pressure via a decrease in cardiac output (17). Acepromazine administration did not have any influence on blood pressure such that SAP, MAP, and DAP values from 20 to 80 min were not significantly lower than at time point ACP.

Both acepromazine and opioids can reduce RR in dogs (15,17). The respiratory effects of acepromazine are associated with its sedative effect and relieving of anxiety (17). Despite causing a reduction in RR, acepromazine did not change blood gases in dogs (17). Administration of opioid analgesics such as fentanyl, result in dose-related respiratory depression in dogs that are awake, as represented by decreased pH and PaO2, and increased PaCO2 (15). Different from other opioid analgesics with high affinity for μ receptors, tramadol was found to cause less respiratory depression than morphine in humans (18) and in dogs, there was no change in RR after IV injection of tramadol at doses as high as 4 mg/kg BW (9). Finding that RR decreased below baseline in all treatment groups at time point ACP and did not change any further after administration of tramadol suggests that acepromazine was the main drug responsible for the decrease in RR in this study.

An indirect rather than a direct method was used for measuring blood pressure, which is a limitation of this study. Oscillometric devices have been used in dogs (19–21), and 1 study reported an acceptable accuracy (21). However, agreement with invasive blood pressure was poor in 2 other studies (19,20). Despite this limitation, in the present study it was aimed to assess the behavior of blood pressure over time and not to report absolute values.

Under the conditions of this study, sedation induced by acepromazine and tramadol was similar to that of acepromazine alone. The main adverse effects of the combination were a decrease in blood pressure and HR, without clinical significance.

**References**


Degree of corneal anesthesia after topical application of 0.4% oxybuprocaine ophthalmic solution in normal equids

Erika Little, Kathy Yvoruchuk-St. Jean, William Little, Fortune Sithole, Guy St. Jean

Abstract

Oxybuprocaine hydrochloride ophthalmic solution has been widely used off-label in horses and donkeys, despite lack of data demonstrating efficacy and safety in these species. The objective of this study was to assess anesthetic efficacy of 0.4% oxybuprocaine hydrochloride ophthalmic solution in horses (n = 5) and donkeys (n = 24) and compare the effects with 0.5% proparacaine hydrochloride ophthalmic solution. The baseline corneal touch threshold (CTT) was measured with a Cochet-Bonnet esthesiometer. Donkeys (n = 12) and horses (n = 5) in group A received sterile ophthalmic solutions 0.4% oxybuprocaine with fluorescein (also termed benoxinate with fluorescein, abbreviated as ben + flu) instilled in one eye and 0.9% sterile sodium chloride solution (NaCl) with fluorescein (Na + flu) in the contralateral eye. Donkeys (n = 12) and horses (n = 5) in group B received sterile ophthalmic solutions (ben + flu) in one eye and 0.5% proparacaine with fluorescein (prop + flu) in the contralateral eye. The CTT was measured at 1 and 5 min post-application and at 5-minute intervals until 75 min after treatment. The CTT changes over time differed significantly between oxybuprocaine-treated and control eyes (P < 0.001). The CTT continued to decrease throughout the duration of the study when compared with baseline values. No statistically significant difference in onset, depth, or duration of corneal anesthesia was found between oxybuprocaine and proparacaine treated eyes during the time of the study. Interestingly, horses were shown to have a significantly more sensitive cornea than donkeys (P = 0.002). Oxybuprocaine and proparacaine reduced corneal sensitivity in donkeys and horses. No local irritation was observed with 0.4% oxybuprocaine.

Résumé

La solution ophtalmique d’hydrochlorure d’oxybuprocaine a été utilisée extensivement en dérogation chez les chevaux et les ânes, malgré le manque de données démontrant son efficacité et son innocuité chez ces espèces. L’objectif de la présente étude était d’évaluer l’efficacité anesthétique d’une solution ophtalmique d’hydrochlorure d’oxybuprocaine 0,4 % chez des chevaux (n = 5) et des ânes (n = 24) et comparer les effets avec une solution ophtalmique d’hydrochlorure de proparacaine 0,5 %. La valeur de base du seuil de contact cornéen (SCT) a été mesurée à l’aide d’un esthésiomètre Cochet-Bonnet. Les ânes (n = 12) et chevaux (n = 5) du groupe A ont reçu une solution ophtalmique stérile d’oxybuprocaine 0,4 % avec de la fluorescéine (également appelée benoxinate avec fluorescéine, abrégé ben + flu) dans un œil et une solution stérile de chlorure de sodium 0,9 % (NaCl) avec de la fluorescéine (Na + flu) dans l’œil contralatéral. Les ânes (n = 12) et chevaux (n = 5) du groupe B ont reçu les solutions ophthalmiques stériles de (ben + flu) dans un œil et de la propacaïne 0,5 % avec de la fluorescéine (prop + flu) dans l’œil contralatéral. Le SCT a été mesuré à 1 et 5 min post-application et à des intervalles de 5 min jusqu’à 75 min après le traitement. Les changements dans le temps du SCT différaient de manière significative entre les yeux traités à l’oxybuprocaine et les témoins (P < 0.001). Le SCT continua de diminuer tout au long de la durée de l’étude lorsque comparé aux valeurs de base. Aucune différence significative dans le début, la profondeur, ou la durée de l’anesthésie cornéenne ne fut trouvée entre les yeux traités à l’oxybuprocaine et la propacaïne durant la durée de l’étude. De manière intéressante, les chevaux avaient une cornée significativement plus sensible que les ânes (P = 0.002). L’oxybuprocaine et la propacaïne ont réduit la sensibilité cornéenne chez les ânes et les chevaux. Aucune irritation locale ne fut observée avec l’oxybuprocaine 0,4 %.

(Traduit par Docteur Serge Messier)

Introduction

Horses and donkeys commonly suffer from ocular injuries, a consequence of the anatomical size and positioning of the eyes accompanied by their inborn “fight or flight” response, which creates a need for further investigation of topical ophthalmic anesthetics in order to select the most effective medication for each case. The cornea is the most sensitive tissue in the body (1). Its sensitivity has been documented by use of an esthesiometer (2,3). The Cochet-Bonnet corneal esthesiometer (CBA) has become the most commonly used esthesiometer in clinical and research settings (1,4). The filament of the CBA becomes increasingly more rigid as it is shortened and exerts an increased pressure on the corneal surface. The corneal touch threshold (CTT) is the minimal amount of corneal stimulation resulting in a consistent blink reflex and is measured by the use of a corneal esthesiometer (5,6). Corneal sensitivity has been measured...
using an esthesiometer in horses (7–9) as well as many other species (9); however, no studies measuring corneal sensitivity were found for donkeys. In previous studies mean corneal sensitivity measured by CTT using a CBA in healthy adult horses has been reported to range from 2.12 ± 0.62 cm to 5.01 ± 0.61 cm (6–8,10–12). The central portion of the equine cornea is the most sensitive as determined by previous studies (7,8).

Commonly used topical ophthalmic anesthetics include: 0.5% proparacaine, 0.5% tetracaine, and 0.4% oxybuprocaine (benoxinate); proparacaine and tetracaine solutions being the most widely used in veterinary ophthalmology (4). Ocular sensitivity manifested by symptoms of acute conjunctival hyperemia, chemosis, and nictitans protrusion has been reported in dogs following topical application of tetracaine (4,13). In dogs, 0.4% oxybuprocaine hydrochloride (benoxinate) is appropriately suited as a topical ocular anesthetic agent that can be used in many clinical settings with few risks of conjunctival changes compared to tetracaine solution (14,15). Its anesthetic effects are comparable in depth and duration to that of proparacaine solution (16,17). In the United States, proparacaine solution is a common topical anesthetic solution used in veterinary practice (4,18). In the dog, studies have shown that significant anesthetic effects on the cornea can be measured for 45 min after the instillation of proparacaine (15) as well as oxybuprocaine solutions (14). The use of proracaine has been evaluated in many veterinary species and has also been proven to be a safe and effective corneal anesthetic agent in cats (16), dogs (9), and horses (6,11).

The rationale for this study was to determine the degree of corneal anesthesia provided by 0.4% oxybuprocaine with fluorescein (ben + flu) ophthalmic solution compared to 0.5% proparacaine with fluorescein (prop + flu) solution in donkeys and horses in addition to evaluating ben + flu for adverse effects in horses and donkeys. This study also allowed for comparison of response of ben + flu solution between donkeys and horses. The hypothesis was that a single topical application of ben + flu or prop + flu solution would provide similar anesthetic depth, but ben + flu would elicit longer anesthetic duration compared to 0.5% proparacaine solution in clinically normal donkeys and horses. It was anticipated that both donkeys and horses would respond similarly to the topical application of ben + flu solution.

Donkeys were randomly divided into 2 groups of 12 for the study. Each horse was used in both group A and group B with a 2-week washout period between treatments. All animals were university-owned and were housed on-pasture throughout the duration of the study. Animals were brought into the same treatment room for the study days to ensure constant ambient temperature, relative humidity, and elimination of environmental artifacts. All animals were considered healthy based on physical examination findings. Only ophthalmologically normal donkeys and horses, demonstrating a Schirmer Tear Test (Schirmer tear test strips; Schering-Plough Animal Health, Union, New Jersey, USA) value > 10 mm/min, absence of corneal fluorescein stain (Bio-Glo fluorescein sodium ophthalmic strips; HUB Pharmaceuticals, Rancho Cucamonga, California, USA) retention, and absence of active corneal or adnexal disease were included in the study. Animals were monitored for adverse effects including blepharospasm, epiphora, and chemosis throughout the study and the investigators, upon completion of the procedure, performed fluorescein staining of the cornea. Blepharospasm, epiphora, and conjunctival hyperemia were graded on a scale from 0 to 3 for each of the criteria evaluated. Grade 0 was considered normal, grade 1 mildly affected, grade 2 moderately affected, and grade 3 severely affected.

### Procedures

All animals in the study were randomly allocated to treatment order. For each animal the baseline CTT was determined in both eyes by use of a Cochet-Bonnet esthesiometer (Cochet-Bonnet esthesiometer; Luneau Ophtalmologie, Chartres, France) immediately prior to topical administration of solutions. All measurements were obtained with minimal restraint and without the use of sedation.

One investigator applied CBA to the cornea while another investigator visualized the application of the esthesiometer while wearing head loupes with a 4× magnification.

All solutions were drawn up in a sterile fashion using a new sterile 25-gauge needle and 1-mL syringe. A new sterile fluorescein strip was used to create the saline with fluorescein and the proparacaine with fluorescein. In order to administer the treatments, 0.2 mL of the selected drug solution was drawn into a 1-mL syringe via a 25-gauge needle. The needle was broken off at the hub and the drug solution was gently sprayed onto the corneal surface (6,11). The same bottle of each drug solution was used throughout the study period and stored according to the manufacturer’s guidelines. In group A, CTT results were compared after topical administration of 0.9% sterile sodium chloride solution (NaCl) with fluorescein (Na + flu) solution in 1 eye and topical administration of ben + flu (Fluorescein sodium and benoxinate hydrochloride ophthalmic solution, USP 0.25%/0.4%; Bausch & Lomb, Tampa, Florida, USA) in the contralateral eye. Corneal touch threshold measurements were obtained immediately after baseline for Group A, 0.2 mL of ben + flu was

### Materials and methods

### Animals

Twenty-four healthy adult castrated male Abyssinian donkeys ranging from 4 to 12 y of age (mean: 6.8 y, SD ± 2.47 y) and 5 healthy adult horses (3 geldings and 2 mares) ranging from 10 to 32 y in age (mean: 21.6, SD ± 8.1 y) were used in the study. Horses were mixed breed Quarter Horse and Thoroughbred.

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administered in 1 eye and 0.2 mL of Na + flu was administered in the contralateral eye. Investigators were masked as to which solution was being placed in which eye. In group B, CTT results were compared after a topical administration of ben + flu was instilled in one eye and a topical administration of prop + flu solution was instilled in the contralateral eye. For all animals, CTT was measured at time 0 (baseline), at 1 min, and 5 min, continuing every 5 min until 75 min after medications had been administered. Following data collection, the cornea was re-stained with fluorescein dye to ensure no corneal damage was sustained. The protocol used for this study was approved by the Institutional Animal Care and Use Committee of the Ross University School of Veterinary Medicine.

Measurement of corneal sensitivity

The CBA was used to measure the sensitivity of the central portion of the cornea. It has a 0.12 mm diameter flexible monofilament nylon thread which has a range of 5 to 60 mm in length to control the intensity of corneal pressure applied by the monofilament. The length of the nylon filament corresponds to pressures of 11 to 200 mg/0.0113 mm², respectively. The CBA is calibrated by the manufacturer and the length of exposed filament determines the pressure exerted on the cornea. The CTT is the mean filament length in mm at which a consistent blink response (3 out of 5 stimulations) is elicited. The CTT readings in mm can be converted to applied force measurements using mg per S where S is 0.0113 mm² of sectional area of the filament. The conversion of measurements made with the esthesiometer (Luneau SAS, Chartres Cedex, France) is shown in Table I. To determine corneal sensitivity, the filament of the esthesiometer was advanced toward the globe and applied perpendicular to the central portion of the cornea. The filament was pressed to the cornea until slight deflection of the filament was evident. The filament was initially applied at 60 mm (maximum length) and decreased in 5 mm increments until a consistent blink was elicited. Investigators wore 4 × binocular loupes to improve accuracy of detection of filament bending. The CTT, or corneal sensitivity, was recorded as the length of the esthesiometer filament that induced a blink reflex for at least 3 of 5 stimulations for a specified filament length (14). Two investigators observed each treatment and concurred with all results.

Treatments

Using sterile technique, fluorescein stain was added to the 0.5% proparacaine solution (Proparacaine hydrochloride ophthalmic solution; Akorn, Lake Forest, Illinois, USA) and to the 0.9% NaCl by a licensed veterinary technician to create a color consistent with that of the ben + flu solution. This was done to mask investigators as to which solution was placed in the eye. After obtaining the baseline CTT for each eye, ben + flu solution, prop + flu solution, or Na + flu was applied to the eye. For Group A, ben + flu was instilled in 1 eye

Figure 1. Donkeys: 0.4% oxybuprocaine versus 0.5% proparacaine.

Figure 2. Donkeys: 0.9% sodium chloride (NaCl) versus 0.4% oxybuprocaine.

Figure 3. Horses: 0.4% oxybuprocaine versus 0.5% proparacaine.

Figure 4. Horses: 0.9% sodium chloride (NaCl) versus 0.4% oxybuprocaine.
and Na+ flu was instilled in the contralateral eye. For Group B, ben + flu was instilled in one eye and prop + flu solution was instilled in the contralateral eye. For each eye in all animals, the CTT value was determined at 1 min, at 5 min, and at 5-minute intervals until 75 min following administration of solution (Figures 1 to 4).

Evaluation of potential adverse effects

Throughout the duration of the study, animals were monitored for evidence of blepharospasm, conjunctival hyperemia, and epiphora. Immediately after CTT measurements were obtained, all corneas were stained with fluorescein stain and evaluated with an ophthalmoscope (Ophthalmoscope; Welch Allyn, Skaneateles Falls, New York, USA) to identify any injury to the cornea during the procedure.

Statistical analysis

Repeated measures analysis of variance (ANOVA) models were run to account for the clustering of CTT measurements of one eye over time for each of the treatments. Each ANOVA model (for each treatment comparison) had CTT as the outcome and time, treatment, and animal identification as factors. For each treatment in a treatment comparison group (in both horses and donkeys) a univariate ANOVA model was run to compare mean CTT measured at time 0 (baseline) and time 1 (1 min after treatment). A P < 0.05 was considered significant.

Statistical software (Stata statistical software, Version 13; Stata Corp, College Station, Texas, USA) was used to perform the ANOVA analysis.

Results

There was no evidence of conjunctival hyperemia, blepharospasm, or epiphora noted at any time throughout the study (Grade 0 out of 3). All eyes were evaluated for corneal injury immediately after data collection using fluorescein staining and ophthalmoscope evaluation.

Minimal adverse ocular effects in this experimental protocol were noted in a single donkey. This animal developed a mild superficial corneal abrasion associated with fructious behavior resulting in the handling of the esthesiometer instrument abrading the cornea. The corneal abrasion was treated topically with an ophthalmic triple antibiotic ointment (Neomycin and Polymyxin B sulfates and bacitracin zinc ophthalmic ointment; Akorn, Lake Forest, Illinois, USA) every 6 h after the procedure. No stain uptake was noted by 36 h post-abrasion, at which time treatment was discontinued. The CTT data from this animal were eliminated from statistical analysis due to the development of the corneal abnormality.

Oxybuprocaine (benoxinate) compared to saline

Oxybuprocaine (benoxinate) solution significantly decreased CTT in both donkeys (P < 0.001) and horses (P < 0.001) versus the controls (Na+ flu). Means +/- SD, with associated P-values, are included in Table I. Significant differences (P < 0.01) were determined by ANOVA for mean CTT values between time 0 and 1 min, as well as all other time points post-administration of ben + flu, while no significant differences were noted between any of the post-administration times. The ANOVA models in both species showed no significant difference in mean CTT between baseline and any time points, nor within post-administration times for the control groups (P = 0.184 donkeys and P = 0.854 horses). In this study, several donkeys and horses were noted to reach an individual CTT of 0 mm (no blink response at a filament length of 5.5 mm) for several min with ben + flu.

Oxybuprocaine (benoxinate) compared to Proparacaine

Compared with baseline values, CTT was significantly decreased (P < 0.01) by 1 min after treatment in the ben + flu and prop + flu treated eyes for both donkeys and horses. Donkeys showed no significant difference (P = 0.60) between ben + flu treated eyes and prop + flu treated eyes at any time point. There was a statistically significant difference (P = 0.001) between the 2 anesthetic solutions in horses. There were no significant differences in the CTT between donkeys and horses relating to ben + flu (P = 0.0983) or prop + flu (P = 0.2435). This study was unable to assess duration of medications as no animal had returned to baseline values by the end of the evaluation period.

Donkeys compared to horses

Horses were shown to have a significantly more sensitive cornea (P = 0.002) than donkeys at baseline (Mean CTT = 5.4 +/- 0.68 cm and 4.22 +/- 1.09 cm respectively). The CTT was not significantly different between donkeys and horses receiving ben + flu solution (P = 0.0983) or prop + flu solution (P = 0.2435).

Figures plotting the means over time for each group are included, and were generated using a commercial spreadsheet program (Excel; Microsoft Office, https://products.office.com/en-us/excel).

Discussion

This study assessed and compared efficacy of corneal anesthesia after application of ben + flu and prop + flu ophthalmic solutions to normal eyes of horses and donkeys. To the author’s knowledge, this is the first study to evaluate the efficacy of topical ophthalmic administration of 0.4% oxybuprocaine (benoxinate) hydrochloride and compare efficacy between ben + flu and prop + flu solutions in the eyes of healthy donkeys and horses. In this study ben + flu solution significantly decreased CTT in donkeys (P < 0.001) and in horses (P = 0.0001) versus the controls. In donkeys, no significant difference was noted between ben + flu treated eyes and prop + flu treated eyes (P = 0.60); however in horses, a statistically significant difference was noted (P = 0.001). In a previous study, the duration of corneal anesthesia in horses using proparacaine solution was 25 min, with maximal corneal desensitization achieved by 5 min (11). In the current study, several donkeys and horses were noted to reach an individual CTT of 0 mm by 1 min post-administration, remaining for several min with both ben + flu and prop + flu solutions. The reason for this finding is unknown but it is possible that behavioral attributes might partially account for the difference. Although unknown, it is possible that the horses and donkeys in this study might be considered more tolerant as they are handled and examined daily by faculty and students. The reason for the apparent difference
in corneal sensitivity between the donkey and horse is unknown. It is possible that a species difference in sensitivity exists. The authors were unable to identify a study examining the differences in sensitivity between these 2 species; therefore, this presents an opportunity for further investigation.

Additional considerations include repeated administration of topical anesthetic as well as volume instilled in each treatment. In a previous study in horses, tetracaine hydrochloride was found to significantly increase the depth and length of corneal anesthesia when administered twice (1 min between treatments) (12). A similar experiment in dogs proved that 1 drop of 0.5% proparacaine hydrochloride ophthalmic solution applied twice with 1 min between doses resulted in significantly increased depth and duration of corneal anesthesia compared with a single drop dosage (15). Although there was a significant reduction in corneal sensation with a single application of ben + flu solution, further studies to evaluate whether or not more complete corneal anesthesia can be achieved with additional applications of the solution (i.e., a second topical administration 1 min after initial treatment) are warranted.

The impact of volume instilled within the eye was not evaluated in this study. The volume instilled, which is approximately equal to 4 drops of solution, is greater than the volume used in studies evaluating the onset and duration of proparacaine solution in cats and dogs (11,15). However, this volume represents the amount typically instilled in clinical application in horses (11).

The Cochet-Bonnet esthesiometer is reliable for measuring corneal sensitivity in normal eyes of horses (1,4), and with exception of one donkey whose behavior created mild injury, resulted in no significant ophthalmic trauma in this study. The application of the Cochet-Bonnet esthesiometer is relatively simple and safe to perform. Limitations of the esthesiometer include subjective interpretation of the response, variations in technique as well as a lack of standardization of deflection pressure (1,6). Variations in humidity and ambient temperature can also alter results by affecting the rigidity of the esthesiometer’s filament (1). Corneal touch threshold measurement was agreed upon by 2 investigators as they concurrently observed the animals’ responses, requiring the animal to blink 3 times out of 5 consecutive applications of the Cochet-Bonnet esthesiometer. This procedure was used in attempt to limit the subjective evaluation of the positive response in the CTT measurements. Standardization of environmental factors was achieved by having all study subjects assessed in the same air-conditioned treatment room. One limitation of this study is that CTT measurements ended prior to all animals returning to baseline values. This precluded comparison of duration of topical anesthesia provided to animals with corneal disease. Use of the medications and the application of the Cochet-Bonnet esthesiometer resulted in no long-term damage in healthy horse and donkey eyes for the length of this study.

In conclusion, the use of ben + flu solution is effective and would pose minimal risk to donkeys and horses with normal corneas.

Acknowledgment

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References


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