Calves are born immunologically naïve and agammaglobulinemic because the bovine placenta does not permit the transfer of maternal antibodies to the fetus prior to parturition. Therefore, it is essential that a newborn calf receive maternal antibodies via colostrum as soon as possible after birth to protect it against disease until its own immune system can mature.

Maternal antibodies help protect neonatal calves against clinical disease caused by BRSV, BHV1, BVDV types 1 and 2, bovine herpesvirus type 1 (BHV1), and parainfluenza virus type 3 at 2 weeks of age (n = 6), 5 weeks of age (6), and both 2 and 5 weeks of age (6) or were assigned to be unvaccinated controls (6). Blood samples were obtained at 1, 2, 5, and 12 weeks for determination of serum neutralization antibody titers against the vaccine viruses, bovine coronavirus, and Mannheimia haemolytica. Antibody rates of decay were calculated.

Calves with initial antibody titers against BRSV < 1:64 that were treated for BRD had a slower rate of anti-BRSV antibody decay than did similar calves that were not treated for BRD. Calves with high initial antibody titers against BRSV and BHV1 had lower odds of BRD than did calves with low initial antibody titers against those 2 pathogens. Vaccination at 2 or 5 weeks of age had no effect on the rate of antibody decay.

CONCLUSIONS AND CLINICAL RELEVANCE
Clinical BRD and the serologic response of dairy calves were associated with initial antibody titers against BRSV and BHV1. Serologic or clinical responses to viral exposure may differ in calves with low passive immunity. (Am J Vet Res 2015;76:239–245)
erations. However, studies conducted to assess the prevalence of these pathogens or their association with the incidence of BRD in calves on commercial dairy farms in North America are lacking. Knowledge of which pathogens are most likely associated with disease in a given population of animals is important for the development of appropriate control and prevention strategies, particularly vaccination programs, for those animals.

The objective of the study reported here was to investigate the effect of clinical BRD and vaccination against BRD on the rate of decay of antibody titers against BRSV, BVDV1, BVDV2, BHV1, PI3, BCV, and *M. haemolytica* in dairy heifer calves ≤ 3 months old. Our hypotheses were that calves with circulating maternal antibodies would not develop detectable antibody responses following clinical BRD or vaccination against BRD and that the rate of antibody decay would vary significantly between calves with and without BRD and between calves that were and were not vaccinated against BRD.

**Material and Methods**

**ANIMALS**

All study protocols were approved by the Animal Care Committee at the University of Guelph and the Institutional Animal Care and Use Committee at the University of Minnesota. A convenience sample of commercial Holstein dairy herds located within a 2-hour radius of the University of Guelph or the University of Minnesota was selected to participate in the study with the informed consent of the herd owners. All calves were home-raised (ie, calves were not sent to a heifer raiser prior to 3 months of age) in accordance with routine farm management practices. For all participating herds, a case of BRD was defined as a calf with an increase in resting respiratory rate, sound, or effort and a fever (> 39.5°C) with at least 1 additional clinical sign such as a cough, nasal discharge, depression, hyporexia, or rough hair coat. Disease events and treatments for individual calves from birth to 3 months of age were recorded and maintained by farm personnel on standardized forms.

**STUDY DESIGN**

To determine the effect of clinical BRD on serum antibody titers against BRSV, BVDV1, BVDV2, BHV1, PI3, BCV, and *M. haemolytica*, a retrospective case-control study was performed. Antibody titers against each pathogen were compared between calves that were and were not treated for BRD. A sample size of 38 calves/group was required to detect a difference of 0.8 in log2 reciprocal antibody titer between calves that were and were not treated for BRD with 95% confidence and 80% power. Thus, 76 unvaccinated calves from 5 herds were enrolled in the study. From each herd, 7 to 10 calves that had been treated for BRD before 3 months of age (cases) were matched with the same number of calves that had not been treated for BRD (controls). Each case calf was matched to a control calf on the basis of date of birth (matched calves were born within 7 days of each other). When possible, case calves that had been treated for BRD between 14 and 46 days of age (the 25th and 75th percentiles for age at first BRD treatment for all calves in the 5 participating herds) were selected for the study, and within a herd, all cases and controls were enrolled in the study in the shortest time period possible.

To determine the effect of vaccination against BRD on antibody titers against BRSV, BVDV1, BVDV2, BHV1, PI3, BCV, and *M. haemolytica*, calves that were part of a larger randomized clinical trial17 were retrospectively selected for evaluation. In that trial,17 a block randomization procedure was used to allocate calves to 1 of 4 treatment groups; calves were administered a multivalent MLV vaccine against BRSV, BVDV1, BVDV2, BHV1, and PI3 (2 mL, IM) at 2 weeks of age (between 15 and 21 days of age; 2-week group), 5 weeks of age (between 35 and 42 days of age; 5-week group), or both 2 and 5 weeks of age (2-dose group) or were administered sterile saline (0.9% NaCl) solution (2 mL, IM) at 2 and 5 weeks of age (unvaccinated control group). All calves were born and raised on 1 farm (herd size, 750 cows) with a high incidence of BRD in calves (44% of calves were treated for BRD prior to 3 months of age during the observation period).17 For the present study, a sample size of 6 calves/group was required to detect a difference of 2 in log2 reciprocal antibody titer among the treatment groups with 95% confidence and 80% power. Thus, 24 calves were evaluated (6 calves in each treatment group [2 week, 5 week, 2 dose, and unvaccinated control]).

**SAMPLE COLLECTION**

From all calves, jugular venipuncture was used to collect a blood sample (10 mL) into a sterile blood collection tube without an anticoagulant at approximately 1 to 7 days of age (enrollment), 15 to 21 days of age (2 weeks), 35 to 42 days of age (5 weeks), and 90 to 120 days of age (12 weeks). Samples were kept chilled until centrifuged at 970 X g for 10 minutes at approximately 20°C. The serum was then harvested from each sample and stored frozen at –20°C until analysis.

**SERUM NEUTRALIZATION**

Serum samples were submitted to the Animal Health Laboratory at the University of Guelph for determination of antibody titers against BRSV, BVDV1, BVDV2, BHV1, PI3, BCV, and *M. haemolytica*. Antibody titers against BRSV, BVDV1, BVDV2, BHV1, PI3, and BCV were determined by use of conventional microtiter virus neutralization assays. Briefly, serum samples were heated at 56°C for 30 minutes. Two-fold serial dilutions of each sample, starting at 1:2 for BRSV, BVDV1, BVDV2, BHV1, and PI3 or 1:4 for BCV were prepared in duplicate in wells of a microtiter plate. Cell culture fluid that contained 100 cell-culture infectious-dose units of the appropriate virus was added to each well in a volume equal to the diluted serum sample. The microtiter plates were then incubated for 1 hour at 37°C (BRSV, BVDV1, BVDV2, BHV1, and
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PI3) or 4°C (BCV), followed by the addition of Georgia bovine kidney cells (BRSV), Madin-Darby bovine kidney cells (BVDV1, BVDV2, BHV1, and BCV), or bovine turbanate cells (PI3) that were suspended in growth medium and supplemented with equine fetal serum (BVDV1, BVDV2, and BHV1) or bovine fetal serum (BRSV, PI3, and BCV), both of which tested negative for BVDV. The plates were incubated at 37°C in a humidified incubator for 3 to 4 days, and then each cell monolayer was visually inspected for characteristic viral cytopathic effects with an inverted microscope. The antibody titer was determined as the 50% endpoint, or the serum dilution at which half of the wells had evidence of cytopathic effects. Known positive and negative serum controls were included for each assay. All samples from an individual calf were analyzed for a given pathogen at the same time to minimize test-to-test variation.

To determine antibody titers against *M. haemolytica*, 96-well plates were coated with a combination of monoclonal antibodies specific for the *M. haemolytica* leukotoxin. Ascites fluid was diluted 1:6,000 in PBS solution (pH 7, 4), and 50 μL of the diluted fluid was added to each well. The plates were incubated at 37°C for 2 hours and washed 4 times (180 μL/well) with a wash buffer that consisted of PBS solution with 0.5% fish skin gelatin and 0.05% Tween 20. The plates were blocked with 60 μL of 5% normal horse serum in PBS solution/well and incubated for 1 hour at 37°C, then washed 4 times as previously described. Lyophilized logarithmic-phase culture supernatant of *M. haemolytica* serotype A1 (American Type Culture Collection 43270) grown in RPMI 1640 medium was reconstituted to 4 times the original concentration in wash buffer, and 50 μL was added to each well. The plates were incubated at 37°C for 30 minutes, then washed as previously described. The serum samples were diluted 1:80 in a sample diluent that consisted of PBS solution with 1.5% Tween 20, 0.5% fish skin gelatin, and 0.3M NaCl. 50 μL of each diluted sample was added to each well, and a 2-fold dilution series of a positive-control serum sample (titer: 1:327,680) obtained from a colostrum-deprived calf that was vaccinated with culture supernatant of *M. haemolytica* serotype A1 was used as a standard on all plates. The plates were incubated at 37°C for 1 hour and washed 4 times as described. A mouse monoclonal antibody against bovine IgG that was conjugated to alkaline phosphatase was added to each well to detect bound antibodies against *M. haemolytica*. The plates were incubated at 37°C for 30 minutes and washed 4 times as described, and then p-nitrophenol phosphate dissolved in diethanolamine buffer was added to each well as a substrate for color development. Optical densities were measured with a plate reader at 405 and 630 nm. Samples with optical densities that did not fall into the linear range determined by the positive-control sample were assayed again at a dilution of 1:20 or 1:320 as required.

**STATISTICAL ANALYSIS**

The reciprocals of the virus neutralization antibody titers were log₂ transformed for analyses, and the *M. haemolytica* antibody titers were likewise calculated and expressed on a log₂ scale. For each calf, the change in antibody titer for each pathogen between sampling intervals was investigated for evidence of seroconversion (≥ 4-fold increase in titer). Descriptive statistics were calculated, and the median titer at the median age of sampling was plotted for each pathogen. A summary statistic approach was used to create a calf-level regression of the slope of antibody titers across the 4 sampling points, by use of the actual age of each calf at the time of sample acquisition. For each pathogen (BRSV, BVDV1, BVDV2, BHV1, PI3, BCV, and *M. haemolytica*), the slope of the regression represented the rate of antibody decay.

The primary variable of interest for the linear regression model was the BRD case or control status for the retrospective case-control study and treatment group (2 week, 5 week, 2 dose, or unvaccinated control) for the trial to determine the effect of vaccination against BRD on antibody titers (vaccination trial). A random effect for herd was included to account for the clustering of calves within herds. The association between the quadratic of the slope for each pathogen and the primary variable of interest was evaluated to assess for curvature of the regression and was not found to be significant, which indicated the rate of decay of the log₂ transformed reciprocal antibody titers was linear for all pathogens. Covariates included in the linear regression model for the retrospective case-control study included the initial antibody titer determined at enrollment (1 to 7 days old) and the interaction between initial antibody titer and the case or control status of the calf. Covariates included in the regression model for the vaccination trial included initial antibody titer, whether the calf was treated for BRD prior to 3 months of age (yes or no), and the respective interactions between those covariates and treatment group. Residuals were assessed to determine whether the model met the assumptions for ANOVA and to evaluate how well the model fit the data. The assumption of normality was also tested. During the regression analysis, covariates with values of *P* > 0.05 were removed by backward elimination in an iterative process, and outliers were included and removed from the model to evaluate their effects on the model outcomes.

For the retrospective case-control study, linear regression was used to assess the associations between the respective initial log₂ reciprocal antibody titers against BRSV, BVDV1, BVDV2, BHV1, PI3, BCV, and *M. haemolytica* and the calf’s case or control status while controlling for actual age at sample acquisition and the inclusion of a random effect for herd. A multivariable logistic regression model was also created to assess the associations between case or control status and the respective initial antibody titers against each pathogen and whether the calf seroconverted to the pathogen in question (yes or no), while controlling for the actual age at sample acquisition and the inclusion
of a random effect for herd. Covariates with values of \( P > 0.05 \) were removed by backward elimination.

For the vaccination trial, the slope of the summary statistic regression for \( \log_2 \) antibody titer per day for the calves in the unvaccinated control group that did not seroconvert was used to calculate the number of days required for a decrease of 1 in \( \log_2 \) reciprocal antibody titer (ie, 1 dilution) in titer and estimate the apparent mean antibody half-life for each pathogen. Thus, the apparent antibody half-life for each pathogen was the reciprocal of the rate of decay of the antibody titer for that pathogen. For example, the apparent antibody half-life, or time required to decrease the \( \log_2 \) reciprocal antibody titer by 1 dilution, would be 20 days for a pathogen with a rate of decay of 0.05 days\(^{-1}\) (ie, 1/0.05 days). All analyses were performed with commercially available statistical software,\(^b\) and values of \( P \leq 0.05 \) were considered significant.

**Results**

**BRD RETROSPECTIVE CASE-CONTROL STUDY**

Of the 76 calves in the retrospective case-control study, 38 (50%) seroconverted to \( \geq 1 \) BRD pathogen between sampling intervals. The majority of those calves seroconverted to *M baemolytica* (25/38 [66%]) and BCV (21/38 [55%]), with only 2 (5%) calves seroconverting to PI3 and 1 (3%) calf each seroconverting to BRSV and BVDV1. All the calves that seroconverted to *M baemolytica* did so between 5 weeks and 3 months of age. Of the 21 calves that seroconverted to BCV, 5 (24%) seroconverted between enrollment and 2 weeks of age, 3 (14%) seroconverted between 2 and 5 weeks of age, and 13 (62%) seroconverted between 5 weeks and 3 months of age. The 2 calves that seroconverted to PI3 did so between enrollment and 2 weeks of age, and those that seroconverted to BRSV and BVDV1 did so between 5 weeks and 3 months of age. Thirteen of 38 (34%) calves seroconverted to more than 1 pathogen (10 calves seroconverted to *M baemolytica* and BCV, 1 calf seroconverted to PI3 and *M baemolytica*, 1 calf seroconverted to BVDV1 and BCV, and 1 calf seroconverted to BRSV and BCV). Seroconversion to any of the BRD pathogens was not significantly associated with a calf being classified as a case. The median \( \log_2 \) reciprocal antibody titers against BRSV, BVDV1, BVDV2, BHV1, PI3, BCV, and *M baemolytica* over time were summarized graphically (Figure 1).

The rate of decay of antibody titers against BRSV for a subset of the BRD cases differed significantly from that of the control calves and was dependent on the initial antibody titer against BRSV (Figure 2). The rate of decay of antibody titer for BRD cases with an initial \( \log_2 \) reciprocal anti-BRSV antibody titer \( \geq 8 \) (ie, the median) did not differ significantly from that of the control calves. However, the rate of decay of antibody titer for BRD cases with an initial \( \log_2 \) reciprocal anti-BRSV antibody titer \( \leq 5.5 \) (ie, the 10th percentile) was significantly slower than that for control calves.

Although the initial antibody titers against BVDV2, BHV1, PI3, and *M baemolytica* were significantly associated with the rates of antibody decay for those pathogens, the respective rates of antibody decay did not differ significantly between BRD cases and control calves. The same was true for antibody titers against BCV, but the assumption of normality was violated for that model, and the application of various transformations to the data failed to improve the model fit. Normality was achieved only when 2 initial \( \log_2 \) reciprocal anti-BCV antibody titers \( > 20 \) (ie, outliers that were > 2 SD from the mean, which might have been indicative of an active BCV infection in the calf or its dam) were removed from the analysis. However, with the outliers removed from the analysis, the initial antibody titer was not significantly associated with the rate of antibody decay. Because of this finding and their undue influence on the model, the outliers were removed from the final model for BCV. For BVDV1, the rate of antibody decay did not differ significantly between case and control calves, and the initial anti-BVDV1 antibody titer was not associated with the rate of antibody decay.

The initial antibody titers against BRSV \( (P = 0.05) \) and BHV1 \( (P = 0.02) \) for case calves were significantly lower than those for the control calves (Table 1).
and were not (controls; 38) treated for BRD. Holstein dairy calves < 3 months old from 5 commercial farms were also exposed to BCV and BHV1, PI3, BCV, and 38 days for antibodies against BVDV2, 22 days for antibodies against BHV1, 21 days for antibodies against PI3, 35 days for antibodies against BCV, and 32 days for antibodies against M haemolytica.

The least squares mean initial log₂ reciprocal antibody titers were calculated by the use of the respective antibody titers against each pathogen measured in serum samples obtained between 1 and 7 days of age while controlling for the actual age of each calf at sample acquisition and the inclusion of a random effect in the model to account for the clustering of calves within herds.

When age at sample acquisition was controlled, the results of the multivariable logistic regression indicated that the odds of being a case were not significantly associated with the initial antibody titer against any pathogen except BHV1 ($P = 0.04$). The odds of being a case increased by 1.5 (95% confidence interval, 1.0 to 2.1) for each decrease of 1 in the initial log₂ reciprocal anti-BHV1 antibody titer.

**Discussion**

Results of the present retrospective case-control study of BRD in dairy calves < 3 months old on 5 farms in Minnesota and Ontario, Canada, indicated that the initial serum antibody titers against BRSV and BHV1 (serum samples obtained between 1 and 7 days of age) were significantly lower for calves that were treated for BRD than for calves that were not treated for BRD, which suggested that BRSV and BHV1 were associated with clinical BRD in the calves on those farms. Despite serologic evidence that calves on those farms were also exposed to BCV and M haemolytica, exposure to those 2 pathogens was not associated with treatment for clinical BRD. The antibody half-lives calculated for the dairy calves of the present vaccination trial were shorter than those reported by investigators of another study that involved beef calves.

In the present case-control study, when the initial antibody titer against BRSV was low, the rate of anti-BRSV antibody decay for calves that were treated for BRD (cases) was slower than that for calves that were not treated for BRD (controls), which suggested that exposure to BRSV was a risk factor for the development of clinical BRD, and calves with a low concentration of maternally derived antibodies against BRSV might have been generating endogenous anti-BRSV antibodies in response to a natural infection. However, the isotype of the anti-BRSV antibodies was not determined in the present study; thus, their origin (maternally derived or endogenous) is unknown. The findings of the present study supported those of other studies that suggest young calves usually do not seroconvert (develop a ≥4-fold increase in antibody titer against a specific pathogen) following viral antigen exposure via MLV or killed vaccines when maternal antibody concentration is high, but are capable of seroconversion in the absence of maternal antibodies. Unlike those other studies, the calves of the present study were not deprived of specific antibodies,

**Table 1**—Least squares mean initial log₂ reciprocal antibody titers against BRSV, BVDV types 1 (BVDV1) and 2 (BVDV2), BHV1, PI3, BCV, and Mannheimia haemolytica leukotoxin for Holstein dairy calves < 3 months old from 5 commercial farms in Minnesota and Ontario, Canada, that were (cases; n = 38) and were not (controls; 38) treated for BRD.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Cases</th>
<th>Controls</th>
<th>Difference (SE) between cases and controls</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRSV</td>
<td>7.5</td>
<td>8.2*</td>
<td>0.68 (0.30)</td>
<td>0.03</td>
</tr>
<tr>
<td>BVDV1</td>
<td>10.1</td>
<td>10.2</td>
<td>0.01 (0.29)</td>
<td>0.58</td>
</tr>
<tr>
<td>BVDV2</td>
<td>9.1</td>
<td>9.7</td>
<td>0.35 (0.36)</td>
<td>0.33</td>
</tr>
<tr>
<td>BHV1</td>
<td>9.2</td>
<td>6.9*</td>
<td>0.70 (0.31)</td>
<td>0.02</td>
</tr>
<tr>
<td>PI3</td>
<td>8.5</td>
<td>9.7</td>
<td>0.14 (0.29)</td>
<td>0.62</td>
</tr>
<tr>
<td>BCV</td>
<td>27.9</td>
<td>24.7</td>
<td>1.0 (0.43)</td>
<td>0.16</td>
</tr>
<tr>
<td>M haemolytica</td>
<td>16.0</td>
<td>16.4</td>
<td>0.32 (0.22)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

The least squares mean initial log₂ reciprocal antibody titers were significantly lower for calves that were not treated for BRD than for calves treated for BRD. The antibody half-lives calculated for the dairy calves of the present vaccination study were shorter than those reported by investigators of another study that involved beef calves.
and initial anti-BRSV antibody titers as high as 1:64 did not prevent the calves treated for BRD from mounting an apparent humoral response. A similar interaction between the effect of disease and initial antibody titer on the rate of decay of that antibody titer was reported by investigators of another study in which dairy calves were vaccinated with a killed BVDV vaccine at 15 days of age and an MLV BVDV vaccine at 45 days of age.

Detection of seroconversion or a differential decline in antibody titers is more difficult for calves with high concentrations of maternal antibody titers than for calves with low concentrations of maternal antibody titers. The antibody concentration must double to achieve an increase of 1 in log₂ reciprocal antibody titer. Therefore, calves with high concentrations of maternal antibodies must produce more endogenous antibodies to achieve an increase of 1 in log₂ reciprocal antibody titer than calves with low concentrations of maternal antibodies. Consequently, endogenous antibody production (eg, seroconversion) might be detectable in calves with low concentrations of maternal antibody titers but not in calves with high concentrations of maternal antibody titers, even though the actual amount of endogenous antibody produced was the same for both groups of calves.

The initial antibody titers against BRSV and BHV1 for the calves of the present case-control study were associated with treatment for BRD before 3 months of age. Calves with high initial antibody titers against BRSV and BHV1 were less likely to be treated for BRD before 3 months of age than were calves with low initial antibody titers against those 2 pathogens. Results of studies conducted in Europe have implicated BRSV, PI3, and BHV1 in the development of BRD in young dairy calves. In North America, BVDV, and M haemolytica have been associated with clinical BRD in young dairy calves. In another study, a small group of dairy calves fed maternal colostrum or a colostrum replacement product had high concentrations of antibody titers against BRSV, BVDV1, BVDV2, BHV1, and PI3, and the mean time for those calves to become seronegative for those pathogens ranged from 3.75 to 6.5 months of age. However, that study did not assess the association between antibody titers and clinical BRD. To our knowledge, little is known about the pathogens that commonly cause BRD in young calves on commercial dairy farms in North America.

Results of multiple studies indicate that young calves vaccinated when maternal antibodies are present fail to seroconvert against the vaccine pathogens. Investigators of other studies report that vaccination of young calves increases the apparent half-life of maternal antibodies and the time required until antibody titers were no longer detectable. Those studies differed from the present study in that the study calves were much older (60 to 90 days) when vaccinated and more likely to develop a humoral response, and the sample size was larger, which improved the researchers’ ability to detect a statistical difference.

In the present vaccination trial, calves vaccinated with a multivalent MLV vaccine did not develop a detectable antibody response, and cell-mediated immunity was not evaluated. In a concurrent study, the cell-mediated responses of calves from the same population that were vaccinated in the same manner as the calves of the vaccination trial were evaluated with a BHV1-specific CD25 expression flow cytometry assay. In that study, calves vaccinated at 2 weeks and at both 2 and 5 weeks had cell-mediated responses at 54 to 66 days of age that were significantly higher than those of unvaccinated calves and calves vaccinated at 5 weeks of age. However, the association of a cell-mediated response with clinical BRD was not assessed in that study, and the antibody titers did not differ significantly among the 4 treatment groups.

In the present study, the rate of antibody decay in young dairy calves was not associated with exposure to most of the putative pathogens of BRD. Calves with low initial concentrations of maternal antibodies that were treated for BRD had a lower rate of anti-BRSV antibody decay than did similar calves that were not treated for BRD. Calves with high initial antibody titers against BRSV and BHV1 were less likely to be treated for BRD during the first 3 months after birth than were calves with low initial antibody titers against those 2 pathogens. These results suggested that BRSV and BHV1 were associated with the development of clinical BRD in this population of young dairy calves, whereas BVDV1, BVDV2, PI3, BCV, and M haemolytica were not associated with clinical BRD in the same population. Vaccination of dairy calves with an MLV vaccine against BRSV, BVDV1, BVDV2, BHV1, and PI3 at 2 weeks of age and at both 2 and 5 weeks of age had no apparent effect on the rate of decline of antibody titers against any of the vaccine pathogens. High concentrations of maternal antibodies in those calves likely impeded the humoral response elicited by vaccination. In young dairy calves, a humoral response subsequent to vaccination is more likely to be detected in calves with low concentrations of maternal antibodies than in calves with high concentrations of maternal antibodies.

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Footnotes
b. BD Vacutainer, Becton, Dickinson and Co, Franklin Lakes, NJ.
c. NADL strain in Mardin-Darby bovine kidney cells, Institute of Armand Frappier, Montreal, QC, Canada.

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