Immunologic responses in corn snakes (Pantherophis guttatus) after experimentally induced infection with ferlaviruses

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OBJECTIVE
To measure immunologic responses of snakes after experimentally induced infection with ferlaviruses.

ANIMALS
42 adult corn snakes (Pantherophis guttatus) of both sexes.

PROCEDURES
Snakes were inoculated intratracheally with genogroup A (n = 12), B (12), or C (12) ferlavirus (infected groups) or cell-culture supernatant (6; control group) on day 0. Three snakes from each infected group were euthanized on days 4, 16, 28, and 49, and 3 snakes from the control group were euthanized on day 49. Blood samples were collected from live snakes on days –6 (baseline), 4, 16, 28, and 49. Hematologic tests were performed and humoral responses assessed via hemagglutination-inhibition assays and ELISAs. Following euthanasia, gross pathological and histologic evaluations and virus detection were performed.

RESULTS
Severity of clinical signs of and immunologic responses to ferlavirus infection differed among snake groups. Hematologic values, particularly WBC and monocyte counts, increased between days 4 and 16 after infection. A humoral response was identified between days 16 and 28. Serum IgM concentrations increased from baseline earlier than IgY concentrations, but the IgY relative increase was higher at the end of the study. The hemagglutination-inhibition assay revealed that the strongest reactions in all infected groups were against the strain with which they had been infected. Snakes infected with genogroup A ferlavirus had the strongest immune response, whereas those infected with genogroup B had the weakest responses.

CONCLUSIONS AND CLINICAL RELEVANCE
Results of this experimental study suggested that the ferlavirus strain with the highest virulence induced the weakest immune response in snakes. (Am J Vet Res 2017;78:482–494)

lung infections in snakes are an important cause of disease and, in most circumstances, are characterized by a chronic or lethal course. Ferlaviruses, belonging to the family Paramyxoviridae, are associated with highly pathogenic outbreaks of respiratory disease in snakes. Respiratory signs and neurologic disorders but also nonspecific clinical signs are commonly observed in affected snakes. Affected snake species include members of the families Viperidae, Colubridae, Elapidae, Crotalidae, Boidae, and Pythonidae. Despite the importance and impact of ferlavirus infection, few reports exist regarding the effects of infection on the immune system of reptiles. In general, the immune system of reptiles is similar to that of mammals, with some differences. It is differentiated into an innate and an adaptive immune system, with the innate response of reptiles being stronger than that of mammalian systems. A first quick but nonspecific response to infection is provided by nonspecific leukocytes, antimicrobial peptides, lysozymes, cytokines, chemokines, and—described only in tortoises—complement.

The adaptive immune system provides a specific response through a cell-mediated system as well as a humoral system. Depending on the species, the intensity of the cell-mediated response of reptiles follows a seasonal rhythm. The humoral response is dominated by immunoglobulins. Antibody types IgM and IgY are described as functionally important, with IgM being the first response that appears to be more prominent in reptiles than in mammalian species. In general, the humoral response is slower in reptiles than in mammals, although little information is available regarding this response. In some...
reptilian species, an alternative antibody response (so-called natural antibodies) exists, which might be of relevance to compensate for this late immune response; these antibodies have been detected in alligators,25 water pythons,36 and garter snakes.27 However, the exact role of natural antibodies remains unclear and needs to be evaluated further.

The most common methods used for the measurement of the humoral immune response against specific viruses are the virus neutralization test and the HI assay. The HI assay is predominantly used for the detection of anti-ferlavirus antibodies.4,5,28,29 Another serologic method described for use in reptile medicine is the indirect ELISA.50 In reptiles, this method was first used in 1995 to demonstrate IgY and IgM in 2 green turtles (Chelonia mydas) following inoculation with bovine serum albumin.31 This method has further been described for use in green turtles (immunization with chicken egg-white lysozyme)22 and tortoises (detection of IgY against 2 herpesvirus isolates)23 as well as for the diagnosis of ophidian paramyxovirus (an earlier name for ferlaviruses)32 and Cryptosporidium infection in various snake species.33 An antibody response in Argentine boa constrictors (Boa constrictor occidentalis) has been identified after immunization with 2,4-dinitrophenyleated bovine serum albumin.34

Two studies provide some basic information on the immune responses of snakes to ferlavirus infection. One of these studies35 involved detection of pulmonary lesions following intratracheal inoculation of 5 Aruba Island rattlesnakes (Crotalus unicolor) and the description of the inflammatory reaction of the lung tissue by use of electron microscopy. In another study,36 the antibody response was evaluated in Western diamondback rattlesnakes (Crotalus atrox) after inoculation with an inactivated paramyxovirus. Use of an HI assay in that study allowed detection of antibody against paramyxovirus beginning on day 42 after a triple vaccination involving different adjuvants. According to the investigators, the serologic test methods used might have been insufficient for exact determination of HI titers of antibody against paramyxovirus, and the amount of antigenic material used for inoculation might have been insufficient. They suggested development of an ELISA as a possibly more sensitive method.

The purpose of the study reported here was to examine specific immunologic reactions of snakes following experimentally induced infections with various ferlavirus strains, with corn snakes (Pantherophis guttatus) as a model species. Special emphasis was given to the hematologic reactions and antibody responses as measured by use of different serologic test systems and antigens.

**Materials and Methods**

**Animals**

Forty-two adult corn snakes (21 males and 21 females) were included in the study. Size of the snakes ranged from 104 to 150 cm, and body weight ranged from 219 to 761 g. All snakes were acquired from a retail pet source and underwent a thorough health examination prior to inclusion in the study. Snakes were also examined for ecto- and endoparasites, and swab specimens from the choana and cloaca and tracheal wash samples were examined for bacterial and fungal pathogens by use of standard aerobic cultivation methods.37,38 In addition, a cloacal swab specimen combined with fluid from a tracheal wash sample was tested for the presence of ferlavirus by use of an established protocol.39 Only healthy snakes without remarkable findings were used for the study.

Snakes were housed in groups of 6 in terraria (approx 140 X 78 X 65 cm) that included ground material, hiding places, and a water basin. Environmental temperature and humidity were kept within a range suitable for the species (20° to 32°C and 50% to 70% relative humidity). The same experimental setup and snakes as used in this study were also used in a study involving comparison of detection rates of ferlavirus infection among various methods, and those results have been published elsewhere. The study protocol was approved by the local animal protection authority (animal trial No. TVV 61/13).

**Ferlavirus isolates**

Three virus isolates belonging to the 3 ferlavirus genogroups described for squamates were used. The first, isolate Xeno-USA99 of genogroup A, had been isolated from a flathead knob-scaled lizard (Xenosaurus platyceps) that lacked specific clinical signs of infection.41 The second, isolate Crot-GER03 of genogroup B, had been isolated from a timber rattlesnake (Crotalus borridus) following a fatal outbreak in a collection of various snake species.41 The third, isolate PanGut-GER09 of genogroup C, had been isolated from a corn snake during a disease outbreak in a corn snake collection.42 All virus strains were cultured on vipers heart VH2 cells in accordance with an established protocol.42 The virus suspensions were each cultured to concentrations of at least 10^6 TCID50/mL and were stored at ~80°C before use in the study.

**Experimental protocol**

The experimental protocol has been described elsewhere.40 Briefly, snakes were allowed to acclimate to the study environment for a 6-day period before experiments began. Snakes were randomly assigned by means of a simple randomization method (letters and numbers from a box) to receive a suspension containing ferlavirus genogroup A (Xeno-USA99; n = 12), B (Crot-GER03; 12), or C (PanGut-GER09; 12; infected groups) or VH2 cell-culture supernatant (control group; 6). On day 0, snakes were intratracheally inoculated with 1 mL of the assigned substance. After administration, concentrations of the viral suspensions were rechecked and confirmed to be 10^6.5 TCID50/mL for all 3 strains.

**Clinical and necropsy examinations**

All snakes were clinically evaluated on a daily basis, and their general health status and behavior
were assessed, with particular emphasis on detection of respiratory and neurologic abnormalities. On days 4, 16, 28, and 49 after intratracheal inoculation, 3 snakes were euthanized and submitted for necropsy examination. Selection was based on the randomization for group assignment (number). Three snakes from the control group were euthanized on day 49. If a snake had to be euthanized because of severe clinical signs (ie, comatose state, abnormal body position, high-grade purulent secretion from tracheal opening, open-mouth breathing, or dyspnea) or if it died spontaneously, 1 less snake was included in the necropsy protocol for the predetermined examination day.

Following necropsy, several tissue samples were collected from the lung, intestines, kidney, pancreas, and brain and tested for the presence of ferlavirus by means of PCR assay and cell culture as described elsewhere. Gross pathological examination included assessment of all internal organs including the lung, and tissue samples collected from the internal organs were routinely processed for histologic evaluation.

**Blood sample collection**

Blood samples were collected on days –6 (baseline), 4, 16, 28, and 49 from all snakes that remained alive, including those that were subsequently euthanized on those days. The ventral tail vein was used as the main collection site. If necessary, blood samples were collected from the heart ventricle under ultrasonographic guidance in accordance with published guidelines. Gross pathological examination included assessment of all internal organs including the lung, and tissue samples collected from the internal organs were routinely processed for histologic evaluation.

**Blood leukocyte counts**

Leukocyte counts in blood samples were performed by use of the estimation method. At 400X magnification, each of 10 microscopic fields of view was assessed, and all WBCs identified in those fields were counted. The total number of WBCs was then multiplied by 200. White blood cells were further differentiated into heterophils, lymphocytes, and monocytes manually at 1,000X magnification by assessing a total of 100 WBCs within 10 randomly selected fields of view. On the basis of the obtained proportions, differential blood cell counts were calculated as percentages and absolute values (based on the WBC count estimate).

**HI assays**

Hemagglutination-inhibition testing was carried out as described elsewhere. Briefly, thawed plasma samples were heated to 56°C for 30 minutes. Then, 25 µL of plasma was serially 2-fold diluted in sterile physiologic saline (0.9% NaCl) solution in a plate containing 96 wells with U-shaped bottoms. Saline solution was used as a negative control substance. No positive control plasma was available. Twenty-five microliters of virus suspension diluted in saline solution to contain 4 hemagglutinating units (hemagglutinating chicken erythrocytes up to a dilution of 1:4) was added to each well of the 96-well plate (1 row was left virus free as a control sample for spontaneous erythrocyte agglutination). Each plasma sample was tested for antibodies against 4 ferlavirus isolates: the genogroup A (Xeno-USA99), B (Crot-GER03), and C (PanGut-GER09) isolates used for inoculation of snakes in the study and a tortoise strain of ferlavirus (Ther-GER99). The virus with which each snake was inoculated was considered the homologous virus strain.

Plates were incubated at room temperature (approx 20°C) for 30 minutes. Afterward, 50 µL of 1% chicken erythrocyte suspension was added to each well and the plates were again incubated at room temperature for 30 minutes. The plasma dilutions at which hemagglutination was detectable were then determined. The HI titer of antibody against each ferlavirus strain was defined as the inverse of the highest dilution at which no agglutination of erythrocytes was visible macroscopically.

**ELISAs for IgM and IgY**

Thirty milliliters of virus-containing cell medium (for each of the 3 ferlavirus strains) was used for antigen preparation for the ELISA. Cells and cellular debris were removed by centrifugation at 2,000 X g for 10 minutes. To concentrate the virus, the suspension was centrifuged in a swinging bucket rotor at 100,000 X g for 2 hours at 4°C. The supernatant was removed, and the virus-containing pellet was resuspended by gentle agitation in 2 mL of solution containing Tris (50mM; pH, 7.4), NaCl (140mM), and EDTA (5 mM). This virus concentrate (2 mL) was centrifuged through a 20% sucrose cushion at 100,000 X g for 4 hours at 4°C. The pellet was washed in Tris-NaCl-EDTA solution (2 mL/tubule) overnight at 4°C and resuspended the following morning. Each virus suspension was then added to 6.5mM 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate, and the resulting suspension was then concentrated and washed with Dulbecco PBS solution (without Ca²⁺ and Mg²⁺; pH, 7.35) by centrifugal filtration (cut-off, 10 kDa) to prepare viral proteins.

For performance of indirect ELISAs, each well of a microtiter plate was coated with 100 µL of virus antigen at a concentration of 1 µg/mL (determined by use of the Bradford method) diluted in 0.154M NaCl. The virus strain used for inoculation was also used in the ELISAs for each infected snake group. The plates were incubated at room temperature for 60 minutes

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on a microtiter plate mixer⁹ (1 X g). The wells were washed 2 times with 0.154M NaCl and 0.1% Tween 20 (wash solution). A control coating was conducted with bovine casein¹⁰ at a concentration of 1 μg/mL in 0.154M NaCl following the same procedure. Plasma samples (100 μL each) diluted 1:200 in 20mM Tris-HCl (pH, 7.35), 0.154M NaCl, 0.5% casein, and 0.1% Tween 20 (assay dilution buffer) were added to the wells. A positive sample (pooled blood sample from the group) and negative control sample (plasma sample from a confirmed ferlavirus-negative corn snake) were added accordingly.

Plates were incubated for 60 minutes in the same conditions, and all wells were washed 3 times with wash solution. Afterward, 100 μL of horseradish peroxidase⁹-conjugated¹⁰ rabbit-anti-IgM (isolated from corn snakes and carpet pythons) IgG⁹ diluted 1:10,000 in assay dilution buffer was added to each well, and the plates were incubated again for 60 minutes in the same conditions. Wells were washed 4 times with wash solution, then 100 μL of substrate (3mM H₂O₂ and 1mM 5,5′,5′-tetramethyl-benzidine in 0.2M citrate buffer [pH 4.0]) was added to each well. Plates were incubated at room temperature for 8 minutes. Finally, 50 μL of 1M H₂SO₄ was added to each well to stop enzymatic activity. The OD of each well was measured at 450 nm by use of an ELISA plate reader.⁷

For determination of the OD of IgY, the same procedure was used with the difference that snake plasma samples were diluted 1:100, and horseradish peroxidase⁹-conjugated¹⁰ rabbit-anti-IgM (isolated from corn snakes and carpet pythons) IgG⁹ was used. In addition, the incubation period for the colorimetric reaction was only 3 minutes, as supported by the results of preliminary testing.

Because only the OD was determined for the IgM and IgY responses, but no data were available to calculate the actual immunoglobulin concentrations and the linearity between the immunoglobulin concentration and the OD was unknown, a direct comparison of IgM and IgY concentrations was not possible. These values were therefore only compared as relative values to respective baseline values, and their correlation to other variables was assessed. Interassay repeatability of ELISAs and HI assays was tested by use of selected individual samples, yielding comparable results between testing days (data not shown).

Statistical analysis

Statistical analysis was performed with the aid of statistical software.¹⁰ Values of P ≤ 0.05 were considered significant. All data were tested for normality of distribution with the Shapiro-Wilk test. Because most of the results were not normally distributed, all values are reported as medians with interquartile ranges (25th to 75th percentile). The Mann-Whitney U test was used to identify significant differences among assessment points (sample collection days). Correlations between variables were tested by calculation of the Spearman rank correlation coefficient (ρ). Strengths of correlations were assessed in accordance with recommended standards,⁹ whereby a coefficient < 0.3 was considered negligible, 0.3 to < 0.5 was considered low, ≥ 0.5 to < 0.7 was considered moderate, and ≥ 0.7 was considered high.

Results

Animals

Only a few clinical signs were observed following intratracheal inoculation of corn snakes in the 3 infected groups with their assigned strain of ferlavirus; details are reported elsewhere.⁴⁰ Observed signs differed among these 3 groups, depending on the virus strain used. The most severe clinical signs were observed in snakes infected with genogroup B ferlavirus. For this group, the study ended on day 35, by which point 5 snakes had died or were euthanized because of their clinical condition. In contrast, only minimal clinical signs were observed in snakes infected with genogroup A, and most snakes in that group had no apparent abnormalities.

Necropsy findings for lung tissue are reported elsewhere.⁴⁰ They ranged from no abnormalities or only mild histologic evidence of inflammation for snakes infected with genogroup A to severe gross or histolopathologic alterations for snakes infected with genogroups B or C. Results of PCR and cell culture assays for ferlavirus detection are reported elsewhere.⁴⁰ Virus was detected in most (92%) lung tissue samples from infected snakes (and not in samples from control snakes). In all snakes in which ferlavirus was detected in lung tissue samples, at least 1 other organ (intestines, pancreas, kidney, or brain) was also positive for ferlavirus. Three infected snakes were negative for ferlavirus infection in all organs tested, and they had no or only slight pathological changes in the lung.

Blood leukocyte counts

For all infected snakes combined, a general increase from baseline (day –6) was identified in various leukocyte counts between days 4 and 16 (Figure 1; Supplemental Tables S1 and S2, available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.78.4.482). Total WBC counts differed significantly from baseline at all subsequent assessment points. Lymphocyte and monocyte counts were significantly greater than at baseline beginning on day 16. For lymphocytes, no significant difference from baseline was detected at the conclusion of the assessment period (day 49). Heterophil counts initially increased from baseline beginning on day 16, and significant differences from baseline were identified on days 28 and 49.

For snakes infected with genogroup A ferlavirus, an increase in total WBC counts was identified beginning on day 16 after infection (Figure 2). Only in this group, significant differences from baseline in total WBC and monocyte counts were evident at all postinoculation assessment points. In contrast, differences
in heterophil and lymphocyte counts were either not significant or variable.

For snakes infected with genogroup B ferlavirus, total WBC counts as well as number of heterophils, lymphocytes, and monocytes increased from baseline on day 16. From day 16, significant differences from baseline were detected in WBC and heterophil counts, and beginning on day 28, significant differences from baseline were detected in lymphocyte and monocyte counts.

For snakes infected with genogroup C, a hematologic response to infection was also identified initially on day 16; however, only a few differences from baseline in total WBC and monocyte counts were evident.

For snakes in the control group, only 1 significant difference from baseline was identified: an increase in median total WBC count on day 28.

HI assays

For all infected snakes combined, an increase from baseline in HI titers of antibodies against all 3 ferlavirus strains was identified between days 16 and 28 (Figure 1; Supplemental Tables S2 and S3, available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.78.4.482). For days 28 and 49, significant differences from baseline were identified in HI titers for all tested virus antigens except one. For the tortoise ferlavirus strain (Ther-GER99), with which no snakes had been inoculated, a significant difference from baseline was identified only on days 28 and 49.

For snakes infected with genogroup A, HI titers of antibody against the homologous virus strain (Xeno-USA99) increased from baseline between days 16 and 28 (Figure 3). A similar reaction was only identified for antibody against Crot-GER03 (Figure 4). For 2 virus strains (Xeno-USA99 and PanGut-GER09), significant differences from baseline were identified on days 28 and 49, whereas for the other 2 virus strains (Crot-GER03 and Ther-GER99), a significant difference from baseline was identified on day 49 only. The increase in HI titers for antibody against the homologous virus strain was the greatest of all antibody reactions.

For snakes infected with genogroup B, HI titers of antibody against the homologous virus strain (Crot-GER03) increased slightly from baseline between days 28 and 35 (when the last snake was euthanized because of its clinical condition; Figure 3). No increase from baseline in HI titers of antibody against any of the other 2 virus strains was identified (Figure 4). No other significant differences were detected for any of the assessment points or virus strains.

For snakes infected with genogroup C, HI titers of antibodies against the different virus strains increased from baseline beginning at either day 16 or day 28. The strongest increase was evident for antibody against the homologous virus strain (PanGut-GER09; Figure 3). Significant differences from baseline were identified for all HI titers from day 28 onward, except for the results for antibody against the tortoise ferlavirus strain.

Two snakes in the control group had detectable HI titers of antibody against each of the viruses used on each of the days tested, with titers ranging from 16 to 64 against the Xeno-USA99 strain, titers ranging from 64 to 256 against the Crot-GER03 strain, titers of 64 against the PanGut-GER09, and titers ranging from 8 to 16 against the tortoise ferlavirus strain. One other snake had measurable titers (16) against strains Crot-GER03 and PanGut-GER09 on days 4 and 28. Statistical analysis revealed no significant differences within this group among the assessment points (Figure 3).

ELISAs for IgM and IgY

For all infected snakes combined, ODs of antibodies against the ferlavirus strains, as measured in plasma samples via ELISA, increased from baseline beginning on day 16 (IgM) or day 28 (IgY; Figure 1; Supplemental Tables S2 and S4, available at avmajournals.avma.org/doi/suppl/10.2460/ajvr.78.4.482). Significant differences from baseline values were
identified for both IgM and IgY from day 28 onward.

For snakes infected with genogroup A, a marked increase from baseline ODs was detected beginning on day 16, with the IgM response appearing to occur earlier than the IgY response (Figure 3).

For snakes infected with genogroup B, in contrast to the other infected groups, only the ODs of IgM increased from day 28 onward. In this group, no significant increases from baseline ODs were detected.

For snakes infected with genogroup C, an increase from baseline ODs was evident from day 16 onward. However, only the IgM response was significantly different from baseline on day 28. No statistical comparisons were possible for day 49, given that only 2 snakes remained in this group.

Snakes in the control group had an increase from baseline in ODs of IgM in plasma samples and a variable change in ODs of IgY. The same 2 snakes with high HI titers also had an increase in ODs of IgY in plasma samples over the study period, but no significant differences from baseline were identified (Figure 3).

Correlations between hematologic and plasma antibody values

With regard to the hematologic reactions to ferlavirus infection, a negligible negative correlation ($\rho = -0.17$) was identified between total WBC counts and proportions of lymphocytes. For the ELISA measurements, a significant but low, positive correlation ($\rho = 0.40$) was identified between ODs of IgM and IgY in plasma samples. A significant positive correlation was also identified between the ODs measured by ELISA and the plasma titers measured by HI assay for antibody against each of the 4 tested ferlavirus strains. This correlation was stronger for IgY ($\rho = 0.47$ to 0.61) than for IgM ($\rho = 0.21$ to 0.31) and was also stronger in comparisons with HI values for strains Xenous-A99 and Crot-GER03 than in comparisons with HI values for strains PanGut-GER09 and Ther-GER99.

In comparisons between hematologic values and ELISA results, a few significant but negligible positive correlations were identified for correlations of IgM ($\rho = 0.26$) and IgY ($\rho = 0.20$) values with total WBC counts, IgM values with heterophil counts ($\rho = 0.24$), and lymphocyte counts ($\rho = 0.27$), and IgY values with heterophil counts ($\rho = 0.20$). All HI values were correlated significantly and moderately to highly ($\rho = 0.67$ to 0.84) with each other, but not with total WBC counts or the differential cell counts.

Correlations among infected snake groups

A significant moderate negative correlation ($\rho = -0.67$) was identified between total WBC and lymphocyte counts for snakes infected with genogroup C.
but not for the other 2 infected groups. No additional correlations were identified among hematologic variables. The correlation between ODs of IgM and IgY in plasma samples was significant in all infected groups, being strongest in snakes infected with genogroup A ($\rho = 0.68$) and comparably low in snakes infected with genogroups B ($\rho = 0.48$) and C ($\rho = 0.45$).

Results varied for correlations between ODs of IgM and IgY in plasma samples and the plasma antibody titers measured via HI assay. For snakes infected with genogroup A, a low to high positive correlation was identified for the 4 ferlavirus strains tested ($\rho = 0.42$ to 0.80). Correlations were highest with the homologous virus strain (Xeno-USA99) and lowest with the tortoise ferlavirus strain (Ther-GER99). This correlation was particularly high for IgY ($\rho = 0.70$ to 0.80). For snakes infected with genogroup B, this correlation was negative but not significant. For the comparison of the results of the HI assays involving 4 ferlavirus strains for each sample, results for each strain were highly significantly correlated for each snake and assessment point except for 2 comparisons involving the tortoise ferlavirus strain (Ther-GER99); no significant correlation between the results of the HI assay involving Xeno-USA99 and those of HI assay involving Ther-GER99; and also no significant correlation between the results of the HI assay involving Crot-GER03 and those of HI assay involving Ther-GER99. Correlations were highest for snakes infected with genogroup A ($\rho = 0.80$ to 0.99)

Figure 3—Median plasma antibody values at various points for corn snakes inoculated intratracheally with genogroup A (strain Xeno-USA99; n = 12; A), B (strain Crot-GER03; 12; B), or C (strain PanGut-GER09; 12; C) ferlavirus (infected groups) or cell-culture supernatant (control group; 6; D) on day 0. See Figure 1 for remainder of key.
For snakes infected with genogroup A, significant correlations were identified between HI values for Crot-GER03, PanGut-GER09, and Xeno-USA99 and total WBC counts. For snakes infected with genogroup B, a significant low negative correlation (\( \rho = -0.36 \)) was identified between HI values for Xeno-USA99 and total WBC counts. See Figure 1 for remainder of key.

**Discussion**

When results for all infected snakes in the present study were assessed as 1 group, a general immunologic response to infection with various ferlavirus strains was characterized, reflecting the course that might be expected with natural infection with various strains in different snake collections. After infection, the general leukocyte response occurred quickly, with a significant increase from baseline identified as early as day 4. Although little scientific information is available on the leukocytic reaction after infection in reptiles, we found this to be a surprisingly quick reaction. Similar reactions have been described for other species, such as leukocytopenia in chickens and leukocytosis in ducks 3 days after infection with velogenic Newcastle disease virus.30 In the present study, the high viral inoculation dose may have contributed to this rapid leukocyte reaction. Therefore, a similarly rapid response may not occur following natural infections, which presumably involve transmission of substantially lower quantities of virus.

In reptiles, leukocytosis is marked initially by heterophilia as a reaction to inflammation, including secondary (bacterial) infections.12,46 Total blood WBC and heterophil counts of infected snakes in the present study had a similar temporal pattern, including a decrease at the end of the 49-day follow-up period. The most probable explanation is the involvement of secondary bacterial infections, which were identified in some of the study snakes at necropsy. The most common site for bacterial infections in experimentally infected snakes in the present study was the lung, and *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Salmonella* spp were detected in this tissue in several cases (data not shown). The lymphocytic reaction is more complex. In addition to the involvement of lymphocytes in immunoglobulin production, lymphoid depletion reportedly occurs as a consequence of ferlavirus infection, leading to secondary gram-negative bacterial infections.6,51 In the present study, blood lymphocyte and monocyte counts increased significantly from baseline beginning on day 16. The relationship between blood lymphocyte and monocyte concentrations and immunoglobulin production is complex.

Seroconversion in snakes following inoculation with antigen reportedly occurs after 4 to 12 weeks, depending on the diagnostic method used. In one study, snakes vaccinated both with and without an adjuvant (oil emulsion) had evidence of seroconversion via HI assay after 6 to 8 weeks, whereas in another study, an increase in antibody values measured via ELISA was identified 4 to 8 weeks after immunization. For turtles and tortoises, ranges of 6 to 9 weeks have been reported.22,23 These responses may vary with the type of antigen used.

When HI assays were used to assess immunologic responses in the present study, the significant increases in plasma anti-ferlavirus antibody titers beginning on day 28 occurred earlier than previously reported.36 The differences in titers among the different antigens evaluated suggested that serologic differences exist among the genogroups of ferlavirus, with genogroups A and B viruses cross-reacting strongly. This finding also helps to explain the considerable variations identified in HI assay results when identical plasma samples from wild Eastern massasauga snakes (*Sistrurus catenatus catenatus*) were submitted to 3 laboratories in a study, yielding antibody detection rates ranging from 0% to 100%. The fact that the lower number of snakes with an immunologic reaction and the lowest plasma HI antibody titers in the pres-
ent study were against the tortoise ferlavirus strain (Ther-GER99) indicated a certain species specificity and genetic distance between this virus and its tortoise host and the general squamate hosts of other ferlaviruses, although the ferlaviruses do not appear to be strongly host specific.41,53

A limitation in the interpretation of the HI assay results in the study reported here is uncertainty of the specificity of the assay, given that antibody titers were measurable in the plasma samples from 3 snakes in the control group. In 2 of these snakes, the highest measured plasma antibody values were against the genogroup B ferlavirus, with titers ranging from 64 to 256 on all days and a peak identified on day 16. These titers and the consistently high titers measured in 2 snakes but not in the others suggested that those snakes had previous contact with a ferlavirus and not a nonspecific immunologic reaction. Because the study snakes had been obtained as adults from a retail pet source, knowledge about their clinical history was not available. Plasma antibody titers of up to 16 were measured in 1 other snake in the control group, and again the highest titers were against the genogroup B ferlavirus, indicating that a value ≥ 16 may be a necessary cutoff value for interpretation of HI titers of antibody against that particular strain.

When ELISAs were used to assess immunologic responses in the present study, the ODs of various antibodies in plasma samples also increased significantly from baseline, beginning on day 28. This timing was again earlier than that reported for other immunologic studies34,51 involving this detection method in reptiles. Performance of ELISAs is generally complicated, and more technical effort is required than with HI assays, but the sensitivity of ELISAs is reportedly higher than that of virus neutralization tests.23 As for the distinction between IgY and IgM, the OD of IgM increased earlier than that of IgY in the present study, which is consistent with reported information.13,14,55,56

The ODs of antibodies measured in plasma samples via ELISAs started to increase slightly but not significantly earlier and more pronounced than did HI titers of antibodies. This indicated that the ELISA method was more sensitive than the HI assay method, particularly during the early stages of infection, as has been reported.23,34 The results of both types of assays were significantly correlated with each other. Therefore, both methods can generally be recommended for measurement of the humoral response in snakes, but the specificity of the HI assay should be examined more closely considering the results obtained for the control group. Additional studies with a more extended follow-up period are recommended to determine the point after ferlavirus infection at which the peak humoral response is to be expected.

Results regarding differences in the immunologic reactions of corn snakes to the 3 virus strains used for infection in the present study were interesting, particularly considering the differences observed in the virulence of these strains.40 The strongest immunologic reactions were detected in snakes infected with genogroup A ferlavirus (Xeno-USA99). This was observed as a rapid increase in ODs of IgM in plasma samples (beginning on day 4), closely followed by increases in ODs of IgY (beginning on day 16) and HI titers of antibody against the homologous virus strain (beginning on day 28), with exclusively significant reactions on day 4 (monocyte counts and IgM) and a general increase in WBC count. This suggested a primary response of the immune system. Although the virus could be detected in various organs of the infected snakes by means of PCR assay or cell culture, infection with genogroup A ferlavirus (Xeno-USA99) only led to mild clinical signs and lesions.40 Such findings could be ascribed to the apparently lower virulence that was observed for that strain during the study period, indicating that a negative relationship existed between the measured immunologic reaction and the virulence of the virus strain. Furthermore, exclusively in this snake group, a significant correlation was identified between blood total WBC count and ODs of IgM as well as IgY in plasma samples, which suggested a potent immune response. The highest significant correlations and the best detection rate of antibodies were identified in snakes infected with genogroup A ferlavirus. A combination of these factors might have been the reason for the mild clinical signs identified in this trial group. Because the immune defense was established soon after infection, the infection might not have had a chance to progress and therefore cause pathological changes.

In contrast, the group with the fewest significant immunologic reactions was the group infected with genogroup B ferlavirus (Crot-GER03), which has been identified as highly virulent.40 Considered with the findings for snakes infected with mildly virulent genogroup A ferlavirus, this could explain the dramatic course of disease and the severe pathological findings for snakes infected with genogroup B, supported by the negative correlation between the tested immunologic reactions and the virulence of the strain.

In contrast to findings for the other 2 groups of infected snakes, total WBC count in blood samples from snakes infected with genogroup B ferlavirus was negatively (but not significantly) correlated with ODs of IgM and IgY in plasma samples, indicating that despite the increase in amounts of circulating leukocytes, antibody production generally decreased. The reaction of the total leukocyte count may also have been attributable to secondary bacterial infection and not necessarily directly to infection with genogroup B ferlavirus. The low rate of antibody production was more likely to have been a direct result of viral factors. It is speculative but conceivable that this virus may have an inhibitory effect on the immune system, as has been described for other paramyxoviruses.57

Significant hematologic and immunologic reactions in snakes infected with genogroup C ferlavirus (PanGut-GER09) reflected the moderate virulence of
that strain. It was remarkable that the IgY response measured via ELISA as a relative increase in ODs was higher and detected slightly earlier than the IgM response. These findings strongly suggested a memory response and could have indicated that these snakes had already been exposed to a ferlavirus before the study began. Even if this had occurred, it would have been difficult to establish inclusion criteria that would ensure a snake was immunologically naive to this virus. Negative results of PCR and HI assays prior to viral challenge would not necessarily rule out previous exposure because only a tiny amount of memory cells might be in circulation at that point (hence the negative results of HI assays at the beginning of the study) and the virus may have been cleared long ago (hence the negative results of PCR assay at necropsy).

A transient significant change in total WBC counts from baseline was identified on day 28 in the control group. This reaction, which could not be confirmed on the subsequent assessment day, might have represented a nonspecific immunologic reaction to the application of the cell material (without virus). The increase in HI antibody titers of 2 control snakes on day 16 might also have represented an unspecific reaction to the cell material, and we postulate that these 2 snakes were previously exposed to ferlavirus for the previously mentioned reasons. Adverse infection of these snakes during the study was highly unlikely because the control group was completely separated from the infected groups in both time and space, and appropriate hygiene standards were used.

Because of the way the snakes in the present study were obtained (ie, from a retail source and not from specific pathogen-free collections), several individual snakes had anti-ferlavirus antibodies before infection in the present study. Other snakes never developed detectable antibody titers despite infection, whereas others had detectable titers that became undetectable over the study period. Four snakes were seropositive for antibody against ferlavirus at the beginning of the study (day –6), including 2 in the group infected with genogroup A ferlavirus and 2 in the group infected with genogroup B ferlavirus. To determine the influence of these seropositive snakes on the findings, statistical analyses were reperformed without inclusion of their data, for all snakes together as well as for the individual groups. All significant differences that were previously identified (Supplemental Table S2) were confirmed, with the exception of 2 hematologic findings (monocyte counts on day 16 for snakes infected with genogroup B [P = 0.06] and lymphocyte counts on day 28 for snakes infected with genogroup A [P = 0.08]).

The influence of these 4 snakes on the overall outcome of the study was therefore limited. However, their reaction to infection was of special interest. The seropositive snakes were euthanized on different days: 1 snake infected with genogroup B on day 4, 1 snake from each of the 2 infection groups on day 16, and 1 snake infected with genogroup A on day 28. No clinical signs were detected in any of the 4 snakes during the study period. Nevertheless, some pathological changes were detected in the lung. These changes ranged from histologically mild signs of inflammation and no gross pathological abnormalities to severe histologic and gross pathological changes. These snakes may have been naturally immunized against ferlaviruses infection from a previous exposure. This would have meant that the viral inoculation actually represented a challenge study, in which the level of protective immunity (from the previous exposure) was being assessed and could explain the fewer clinical signs and pathological changes in some of them, compared with in the other snakes. Nevertheless, tissue samples from the lung and at least one of the other examined organs yielded positive results of PCR or cell culture assays for ferlaviruses. The measurable HI titers of antibodies in plasma samples from the 4 snakes ranged initially from 8 to 128. These titers (and accordingly the ODs of IgM and IgY in plasma samples as measured via ELISA) remained constant or increased only slightly during the course of infection. In these snakes, no evidence of protection against infection was observed. The snakes that had negative results of antibody assays at the end of the study were all seronegative at the beginning as well; therefore, no connection could be drawn between the initial titer and the course of the infection.

The aforementioned findings for the 4 snakes suggested that although the initial seropositivity was likely an indication of past exposure to ferlaviruses, it did not indicate immune protection against ferlavirus infection or disease. A report exists of high HI titers of antibody against ferlavirus in snakes in which no clinical disease was observed. This is further evidence of a negative correlation between antibody response and pathogenicity. Snakes with persistently high HI antibody titers may also serve as a reservoir for ferlaviruses, particularly given that the present study revealed that virus replication in tissues occurs even in the presence of detectable antibodies. Antibody detection cannot be recommended as an indicator for immunity against ferlaviruses.

Three snakes from 2 infected groups in the present study (the group infected with genogroup A and the group infected with genogroup C) had negative results of PCR and cell culture assays for ferlaviruses at necropsy. One snake (infected with serogroup C) was euthanized on day 28, and 2 other snakes (1 from each group) were euthanized on day 49. These snakes had no evidence of abnormalities or only mild histologic signs of inflammation at necropsy. None had detectable anti-ferlavirus antibodies at the beginning of the study. After intratracheal inoculation with assigned viruses, a distinct humoral immune response (HI antibody titers and ODs of IgM and IgY) was detected beginning on day 4 or 16, with peaks on day 28. Compared with humoral immune responses of the other snakes in the respective infected groups,
responses of these particular snakes were similar, indicating that some snakes were able to combat and clear the infection regardless of the pre-existing antibody against the virus.

In the present study, snakes were obtained from an unremarkable source believed to minimize the risk of prior ferlavirus exposure and randomly assigned to treatment groups. However, because of the nature of the exotic pet market, it can be difficult to obtain a homogeneous group of snakes with identical health and immunologic statuses. Before the study began, all snakes were tested for shedding of infectious agents, particularly ferlaviruses, and only snakes with unremarkable findings were included. Negative results of anti-ferlavirus antibody assays were not inclusion criteria. Previous research has demonstrated that HI titers of antibody against ferlavirus in plasma samples from snakes of various collections are correlated neither with shedding of the virus nor with signs of disease but can be found in those collections without any signs of ferlavirus infection. In addition, snakes shedding ferlavirus can have negative results of HI assays of antibody against ferlavirus. This again suggests that the reliability and specificity of the HI assay are questionable.

Sample collection frequencies and related sample sizes in the present study were limited. The results for day 49 were particularly difficult to evaluate because by that point, the number of remaining snakes was low. A statistical analysis of the results obtained on this day was therefore not possible for the most part. Nevertheless, the large overall number of snakes (n = 36) in the infected groups at study onset allowed relevant statistical conclusions.

The method used for total WBC, heterophil, lymphocyte, and monocyte counts as described by other investigators and used in the present study represents a technique of estimation. Compared with automated blood cell counters validated for use in mammals, this estimation method has some restrictions, such as inaccuracies attributable to technique differences in blood smear preparation. An alternative approach that yields more standardized results, at least for total WBC count, is manual counting by use of a Neubauer-ruled hemocytometer chamber and Natt and Herrick solution. Various methods for obtaining reliable leukocyte counts have been evaluated, and differences have been identified among results obtained. In general, the hemocytometer method has some advantages as it facilitates standardization of the count method, but also some limitations, and concurrent evaluation of a quality control sample with the estimation technique has been recommended when the hemocytometer count method is used. Given these observations, caution should be used when comparing results obtained with different methods. However, within the method, and given a standardized procedure, the estimation method used in the present study can yield reliable results. In the authors’ clinic as well as in most specialized laboratories in Germany, the estimation method has been established as a routine method.

Overall, the study reported here revealed that the immune system of snakes responds much earlier to experimentally induced ferlavirus infection than previously reported. However, the immune response appeared to be influenced by strain virulence, with more virulent strains being associated with a significantly weaker immune reaction. The first signs of an immune response could be identified in the blood leukocyte response, specifically that of total WBCs and monocytes. Therefore, a CBC, even performed by use of the estimation method used in the study, may provide a first, quick indication of an active viral infection until results of more specific tests are available.

A detectable humoral response was evident in corn snakes beginning on day 16, with significant changes from baseline identifiable on day 28. Even though the ELISAs used in the present study appeared to be more sensitive and, therefore, a reliable diagnostic method, the commercially available HI assay also yielded reliable results. A cutoff value of 16 is recommended for interpretation of positive HI results, at least when serogroup B ferlavirus is used as the antigen. In general, the virus antigen used influences HI measurements. For practical diagnosis, in addition to virus detection, combined evaluation of hematologic and humoral immune responses is recommended to obtain additional information on disease development. However, because the immune response depended greatly on the virus strain in the present study, the serologic response cannot be used to draw conclusions regarding the severity of a ferlavirus infection.

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Footnotes

a. Sarstedt, Nümbrecht, Germany.
b. DiffQuik, Dade Behring, Marburg, Germany.
c. Hettich EBA 8S centrifuge, Hettich, Tuttingen, Germany.
d. Techno Plastic Products, Trasadingen, Switzerland.
e. Greiner Bio-One GmbH, Frickenhausen, Germany.
f. Lohman Animal Health, Cuxhaven, Germany.
g. Allegra X-22R centrifuge, Beckman Coulter, Munich, Germany.
h. Ultracentrifuge, Optima L-70K, Beckman Coulter, Munich, Germany.
i. SW 28, Beckman Coulter, Munich, Germany.
j. CHAPS, Sigma-Aldrich Chemie GmbH, Tauferkirchen, Germany.
k. Vivaspin 6, Sartorius Stedim Biotech GmbH, Göttingen, Germany.
l. Corning Costar cell culture plates, 96 well, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.
m. Cytoskeleton Inc, Denver, Colo.
n. IKA Schüttler MTS 2, IKA-Werke, Staufen, Germany.
o. Sigma-Aldrich Chemie GmbH, Tauferkirchen, Germany.
p. HRP, Roche, Mannheim, Germany.
q. BioGenes GmbH, Berlin, Germany.
r. ELISA plate reader anthos ht III, anthos Mikrosysteme, Krefeld, Germany.
s. SPSS Statistics, version 22, IBM, Armonk, NY.
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