Journal of Zoo and Wildlife Medicine 39(3): 358–361, 2008

MEASURING AGREEMENT AND DISCORD AMONG HEMAGGLUTINATION INHIBITION ASSAYS AGAINST DIFFERENT OPHIDIAN PARAMYXOVIRUS STRAINS IN THE EASTERN MASSASAUGA (*SISTRURUS CATENATUS CATENATUS*)

Matthew C. Allender, D.V.M., M.S., Mark A. Mitchell, D.V.M., M.S., Ph.D., Michael J. Dreslik, M.S., Ph.D., Christopher A. Phillips, M.S., Ph.D., and Val R. Beasley, D.V.M., Ph.D., Dipl. A.B.V.T.

Abstract: At present, the hemagglutination inhibition (HI) assay is the sole commercially available serologic method available to detect exposure to ophidian paramyxovirus (OPMV) in snakes. During 2006, 26 eastern massasaugas (*Sistrurus catenatus catenatus*) were collected, and blood was sampled to determine their OPMV status. Samples from each snake were divided into 3 aliquots and tested by using commercially available HI assays against the 4 OPMV isolates used in the 3 laboratories that offer the service. All snakes were tested for antibodies by using HI assays against the green tree python (GTP), San Lucan rattlesnake (SLR), and Aruba Island rattlesnake (AIR) isolates. Twenty-five snakes were tested for antibodies against the western diamondback rattlesnake (WDR) isolate. All samples tested against the GTP and SLR were positive (26/26), whereas 56% (14/25) of the WDR assays were positive, and none (0/26) of the AIR assays yielded a positive result. There was 100% agreement between the GTP and SLR assays, and complete disagreement between the SLR and AIR, as well as the GTP and AIR assays. Kappa statistics for the GTP–WDR, SLR–WDR, GTP–AIR, SLR–AIR, and WDR–AIR indicated that the assays had less than chance agreement. The results demonstrate that current HI assays are not reliable as a sole diagnostic assay in the eastern massasauga. Furthermore, HI assays need to be evaluated by using other parameters to determine OPMV exposure in eastern massasaugas.

Key words: Eastern massasauga, hemagglutination inhibition, paramyxovirus, *Sistrurus catenatus*, snake.

INTRODUCTION

Ophidian paramyxovirus (OPMV) is a potentially lethal disease in snakes, with infected individuals exhibiting a range of clinical signs, including open-mouth breathing, regurgitation, respiratory discharge, pneumonia, and convulsions.[5,7] In captive viperid species, outbreaks are associated with high mortality rates in all age classes.[7,8,10] An outbreak of OPMV in susceptible wild populations of snakes could result in catastrophic mortality that impacts viability, especially if the species involved is threatened or endangered.

Hemagglutination inhibition (HI) is the most common type of antemortem assay for evaluating OPMV disease status. HI can be used to determine past exposure or can be performed serially to confirm active infection. Quarantine protocols at many zoologic and private reptile institutions require negative OPMV titers before release of newly acquired specimens into collections. Future reintroductions of captive snakes into the wild could require a similar seronegative status before release to minimize risks of disease introduction. Assaying free-ranging individuals in populations is needed to assess the frequency and prevalence of OPMV exposures and whether individuals from a particular endemically infected population should be introduced into an immunologically naïve population. Accordingly, reliable methods to define a snake's OPMV status are needed; thus, the importance of assessing currently available methods is a priority. This study was designed to determine the level of agreement among 3 HI assays by using 4 snake isolates from 3 different U.S. laboratories. A population of wild-caught eastern massasaugas (*Sistrurus catenatus catenatus*) was sampled from Illinois. The specific biologic hypotheses were 1) assays from isolates from similar genera would be in good agreement, 2) seroprevalence of OPMV by using all assays would be 100% based on a previous sampling of the population,[1] and 3) snakes with higher titers would have significantly higher total solids concentrations as a result of increased gamma globulins.

From the Department of Small Animal Clinical Science, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, Tennessee 37996, USA (Allender); Departments of Veterinary Clinical Medicine (Mitchell) and Veterinary Biosciences (Beasley), College of Veterinary Medicine, University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61802, USA; and the Illinois Natural History Survey, 1816 South Oak Street, Champaign, Illinois 61820, USA (Dreslik, Phillips). Correspondence should be directed to Dr. Allender (mattallender@utk.edu).

MATERIALS AND METHODS

Study design

Twenty-six wild eastern massasaugas were captured (March to May 2006) from South Shore State Park (38°37′11′′N, 89°18′14′′W) and Eldon Hazlet State Park (38°39′28′′N, 89°19′51′′W) near Carlyle, Illinois (USA). The protocol was approved by the University of Illinois Institutional Animal Use and Care Committee.

Sample collection

Blood samples were collected from the ventral tail vein of each snake and were divided equally into 3 aliquots; however, in one case, the sample collected was sufficient to fill only 2 aliquots. Detailed descriptions of the study sites and sample collection methods were previously reported.[1] To minimize sample handling bias, all plasma samples were prepared and frozen the day they were collected. All samples, on the same day, were batch sent on wet ice via overnight courier to each institution. Confirmation of delivery was obtained the next day from all 3 laboratories. For all HI assays, the reported titer was the last dilution that formed a pellet.

Plasma was submitted to Laboratory 1 for HI testing by using the western diamondback rattlesnake (*Crotalus atrox*) (WDR) OPMV isolate. The OPMV isolate used was collected from the lungs of a snake with pneumonia and was confirmed to be a paramyxovirus based on physiochemical properties and electron microscopy. Plasma from each sample was heat inactivated at 56°C for 30 min, treated with kaolin (25%), and adsorbed to washed guinea pig red blood cells (RBC) at 0.1 μl/ml. Samples were tested by using a standard HI protocol, including titration to 8 hemagglutinating units (HA) of the virus. Titers ≥1:64 were considered indicative of previous exposure. The protocol of the laboratory suggested that, depending on a snake's history, samples with low titers should be retested in 2 to 3 wk.

Plasma was sent to Laboratory 2 for HI testing with an Aruba Island rattlesnake (*Crotalus unicolor*) (AIR) OPMV isolate. The assay was performed as previously reported[6]; however, the addition of bovine serum albumin has since been discontinued by the laboratory (April Childress, pers. comm., 2006). Titers <1:20 were considered negative, 1:40 to 1:80 were suspect, and >1:80 were positive. The laboratory recommended serially testing positive samples at least 4 wk apart to characterize a snake's disease status.

Plasma submitted to Laboratory 3 was evaluated by using a San Lucan rattlesnake (*Crotalus mitchelli mitchelli*) (SLR) and a green tree python (*Morelia viridis*) (GTP) OPMV isolate as described.[2] Plasma was heat inactivated at 56°C and diluted 1:10 in 0.01 M potassium periodate and 0.6% glycerol. Guinea pig RBCs (Lampire Biological Laboratories, Pipersville, Pennsylvania, USA) were washed 3 times in sterile phosphate buffered saline solution (PBS) (Invitrogen, Carlsbad, California 92008, USA), and a 1% dilution was made in PBS. The 2 strains of OPMV used for this assay were titrated and diluted to 8 HA each. Each plasma sample was tested in duplicate. Twofold serial dilutions in PBS were prepared with virus and incubated for 1 hr at room temperature (20–25°C). A 1% suspension of guinea pig RBCs was added, followed by incubation for 1 hr at room temperature. Results were observed after 60 min. Titers that reflected definitive exposure were considered low (1:20 to 1:80), low–moderate (1:160), and moderate (1:320 to 1:640).

Table 1. Results of hemagglutination inhibition assays demonstrating exposure to 4 isolates of ophidian paramyxovirus by using the same eastern massasauga plasma divided equally.

Laboratory no.	Isolate	Positive result
1	Western diamondback rattlesnake	14/25
2	Aruba Island rattlesnake	0/26
3	San Lucan rattlesnake	26/26
3	Green Tree python	26/26

Statistical analysis

The 95% confidence intervals (CI) were calculated for binomial proportions when appropriate. When the sample proportion was 0, the technique described by van Belle and Millard[12] was used. Cohen's kappa test was used to measure the level of agreement between the serostatus (positive or negative) between the different laboratories and isolates.[9] The Kruskal–Wallis test was used to determine if total solid concentrations differed based on seropositivity. Statistical analyses were performed by using SPSS 11.0 (SPSS Inc., Chicago, Illinois 60606, USA).

RESULTS

Results of HI assays are summarized in Table 1. All samples tested against the GTP (26/26) and the SLR isolates (26/26) were seropositive. The samples tested against the WDR were seropositive in 56% (14/25, 95% CI: 37–75) of the cases. All of

the HI assays against the AIR isolates were negative (0/26, 95% CI: 0–11.5). There was 100% agreement between the GTP and SLR assays, and 100% disagreement between the SLR and AIR, as well as between the GTP and AIR assays. The kappa statistics for the WDR–GTP, WDR–SLR, SLR–AIR, GTP–AIR, and WDR–AIR comparisons were all <0.02. Based on the suggestions of Landis and Koch,[9] the kappa for these assays had less than chance agreement. There was no significant difference in total solid concentrations based on serologic titer (SLR: $P = 0.15$; GTP: $P = 0.12$; WDR: $P = 0.45$).

DISCUSSION

Recently, during an initial health serosurvey, OPMV seropositive wild-caught eastern massasaugas in Illinois were identified.[1] The initial conclusion was that snakes were exposed to OPMV in the wild because all of the animals were seropositive. However, cross-reactivity in the assay that resulted in false-positive results and misclassification of disease status was also considered. Nevertheless, the finding of 100% seropositivity for OPMV in an endangered species was alarming, because exposure to the virus could have long-term implications for conservation of this species.

The authors hypothesize that this wild population of eastern massasaugas evaluated would have 100% seropositive reactions with HI to OPMV. The results of this study suggest that the seroprevalence could range from 0–100%, depending upon the isolate examined. The genetic relatedness of these OPMV isolates has not been examined. It is possible that the GTP, SLR, and WDR isolates are more closely related to the strains of OPMV to which the eastern massasaugas of this study were exposed, which resulted in positive titers. Moreover, it seems likely that the AIR isolate is not closely related to the OPMV strains to which the Illinois massasaugas were exposed. Thus, the current state of knowledge regarding OPMV in massasauga rattlesnakes is insufficient to support a long-term health management plan for the eastern massasauga. If the population studied in Illinois has been exposed to OPMV, it may preclude plans to supplement free-ranging populations with captive-bred snakes unless immunity of the transplanted individuals can be conferred before release. Perhaps snakes that are strongly seropositive for the AIR isolate should not be reintroduced to this wild population, yet snakes that are strongly seropositive for the GTP, WDR, and SLR isolates might be safe after release.

Conversely, if this population is truly negative to all isolates of OPMV because of false-positive results, it may be at risk for high morbidity and mortality if any such positive snake is introduced. All commercially available serologic tests currently available to measure immune responses to OPMV are HI assays. HI tests are rapid, easy to perform, and do not require species-specific antibodies. However, inherent limitations of HI assays are cross-reactivity between similar viruses and moderate specificity, which can result in excessive numbers of false-positive samples. Because cross-reactivity may confuse diagnostic efforts, an important research goal will be to determine whether the different viral strains are commensals or pathogens. Regardless of the basis for conflicting findings, whenever repeatability of results among laboratories is low, then more specific assays are needed.

The results of this study reveal an urgent need for studies to determine the sensitivity and specificity of different OMPV HI assays. Validated studies that determine the reactivity of different genetic isolates to current HI isolates are needed. There are numerous isolates of OPMV,[3] and the genetic relatedness and reactivity of the isolates used in the laboratories have not been evaluated in the eastern massasauga. One approach would be to take snakes into captivity followed by in vivo exposure to different isolates of OPMV. Studies might focus on nonthreatened species, in conjunction with very limited studies of massasaugas that are no longer important to the breeding pool. By contrast, if work is to be focused solely on direct studies of eastern massasauga rattlesnakes (a state endangered species), then the capacity to discern the validity of these HI tests will be constrained. In any case, for efficient comparisons of numerous isolates of OPMV, differences in sample handling, technique, test validation, and approaches to interpretation should be minimized.

In addition to HI assays, future research should elucidate other techniques to measure the OPMV status of eastern massasaugas and other snakes, including, when possible, the monitoring of populations in the wild that experience a spontaneous wave of infections. Of importance, future studies of infection status in the wild and in captivity should involve measurement of antibody concentrations and proteomics, with concurrent histopathology, ultrastructural studies, virus isolation, and viral genome sequencing. Both immunohistochemistry and reverse transcriptase–polymerase chain reaction (rt-PCR) assays for OPMVs have been reported.[4,11] These assays require tissue biopsy specimens to identify virus-infected cells. By using such methods, false-negative results may be obtained, unless

the infectious agent is disseminated through the tissues. Also, although the sensitivity of rt-PCR is typically high, false positives can occur from contamination, thus sample handling and preparation must be carefully performed.[13]

Management decisions for massasaugas or other viperid snakes should not depend on the interpretation of a single HI test result but should also account for the history and clinical signs of the individual, the departing population, and the receiving population. Moreover, management will need to rely upon similar comparative studies to evaluate other snake species to determine their responses to HI assays for OPMV. In addition, experimental studies that compare the HI assays to a ''gold standard'' (e.g., viral isolation, viral genomics, antibody sequencing, and Western blot analysis) are needed to establish assay sensitivity, specificity, and positive and negative predictive values. Final recommendations for the management of viperids with regard to OPMV should rely on developing and applying such assays for use in health disease surveys.

Acknowledgments: The authors thank the Morris Animal Foundation for their support with this project (Grant D03Z0-114). We thank Dr. Arturo Angelo from the Texas Veterinary Medicine Diagnostic Laboratory, Ms. April Childress at the University of Florida, and Dr. Melissa Kennedy at the University of Tennessee for their assistance in performing the assays and the descriptions of the methods they used. The efforts put forth by S. J. Baker and A. J. Berger in capturing snakes and obtaining blood samples were greatly appreciated.

LITERATURE CITED

1. Allender, M. C., M. A. Mitchell, C. A. Phillips, K. Gruszynski, and V. R. Beasley. 2006. Hematology, plasma biochemistry, and antibodies to select viruses in wild-caught eastern massasauga rattlesnakes (*Sistrurus catenatus catenatus*) from Illinois. J. Wildl. Dis. 42: 107–114.

2. Burleson, F. G., T. M. Chambers, and D. L. Weidbrauk. 1992. *In:* Virology: A Laboratory Manual. Academic Press, San Diego, California. Pp. 130–134.

3. Franke, J., S. Essbauer, W. Ahne, and S. Blahak. 2001. Identification and molecular characterization of 18 paramyxoviruses isolated from snakes. Virus Res. 80: 67–74.

4. Homer, B. L., J. P. Sundberg, J. M. Gaskin, and J. Schumacher. 1995. Immunoperoxidase detection of ophidian paramyxovirus in snake lung using polyclonal antibody. J. Vet. Diagn. Invest. 7: 72–77.

5. Jacobson, E. R. 1980. Paramyxo-like virus infection in a rock rattlesnake. J. Am. Vet. Med. Assoc. 177: 796–799.

6. Jacobson, E. R., and F. Origgi. 2002. Use of serology in reptile medicine. Semin. Avian Exotic Pet. Med. 11: 33–45.

7. Jacobson, E. R., J. M. Gaskin, D. Page, W. O. Iverson, and J. W. Johnson. 1981. Illness associated with paramyxo-like virus infection in a zoologic collection of snakes. J. Am. Vet. Med. Assoc. 179: 1227–1230.

8. Jacobson, E. R., J. M. Gaskin, S. Wells, B. S. Bowler, and J. Schumacher. 1992. Epizootic of ophidian paramyxovirus in a zoological collection: pathological, microbiological, and serological findings. J. Zoo. Wildl. Med. 23: 318–327.

9. Landis, J. R., and G. G. Koch. 1977. The measurement of observer agreement for categorical data. Biometrics 33: 159–174.

10. Potgieter, L. N., R. E. Sigler, and R. G. Russell. 1987. Pneumonia in Ottoman vipers (*Vipera xanthena xanthena*) associated with a parainfluenza 2-like virus. J. Wildl. Dis. 23: 355–360.

11. Sand, M. A., K. S. Latimer, C. R. Gregory, P. M. Rakich, E. Jacobson, and K. E. Pennick. 2004. Molecular diagnosis of paramyxovirus infection in snakes using reverse transcriptase-polymerase chain reaction and complementary deoxyribonucleic acid:ribonucleic acid in situ hybridization. J. Vet. Diagn. Invest. 16: 442–448.

12. van Belle, G., and S. P. Millard. 1998. STRUTS: Statistical rules of thumb. www.nrcse.washington.edu/research/struts/chapter2.pdf. Seattle, Washington. Pp. 3–13.

13. Wellehan, J. 2006. Understanding diagnostic testing. *In:* Mader, D. M. (ed.). Reptile Medicine and Surgery, 2nd ed. W. B. Saunders Co., Philadelphia, Pennsylvania. Pp. 1062–1067.

Received for publication 12 September 2007