Orthobunyaviruses, a Common Cause of Infection of Livestock in the Yucatan Peninsula of Mexico

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Abstract. To determine the seroprevalence of selected orthobunyaviruses in livestock in the Yucatan Peninsula of Mexico, a serologic investigation was performed using serum samples from 256 domestic animals (182 horses, 31 sheep, 1 dog, 37 chickens, and 5 turkeys). All serum samples were examined by plaque reduction neutralization test using Cache Valley virus (CVV), Cholul virus (CHLV), South River virus (SOURV), Kairi virus, Maguari virus, and Wyeomyia virus. Of the 182 horses, 60 (33.0%) were seropositive for CHLV, 48 (26.4%) were seropositive for CVV, 1 (0.5%) was seropositive for SOURV, 60 (33.0%) had antibodies to an undetermined orthobunyavirus, and 13 (7.1%) were negative for orthobunyavirus-specific antibody. Of the 31 sheep, 6 (19.3%) were seropositive for CHLV, 3 (9.7%) were seropositive for CVV, 4 (12.9%) were seropositive for SOURV, 16 (51.6%) had antibodies to an undetermined orthobunyavirus, and 2 (6.5%) were negative for orthobunyavirus-specific antibody. The single dog was seropositive for SOURV. Four (11%) chickens had antibodies to an undetermined orthobunyavirus, and 1 (20%) turkey was seropositive for CHLV. These data indicate that orthobunyaviruses commonly infect livestock in the Yucatan Peninsula.

INTRODUCTION

The family *Bunyaviridae* comprises the largest group of arthropod-borne viruses (arboviruses) and consists of five genera: *Orthobunyavirus*, *Phlebovirus*, *Hantavirus*, *Nairovirus*, and *Tospovirus*.^{1,2} A characteristic feature of all viruses in the family *Bunyaviridae* is that they possess a tripartite, single-stranded, negative-sense RNA genome.^{2,3} The three genomic segments are designated as small (S), medium (M), and large (L). The genus *Orthobunyavirus* contains 18 serogroups, including the Bunyamwera (BUN) and California (CAL) serogroups. Viruses in the BUN serogroup include Cache Valley virus (CVV), Cholul virus (CHLV) and Kairi virus (KRIV). The CAL serogroup includes South River virus (SOURV), as well as important human pathogens such as La Crosse, Jamestown Canyon and Tahyna viruses.

We recently reported the isolation of 20 orthobunyaviruses from mosquitoes in the Yucatan Peninsula of Mexico in 2007 and 2008.^{4–6} These isolates were identified as CVV (n = 17), CHLV (n = 1), KRIV (n = 1), and SOURV (n = 1). Cache Valley virus is the best characterized of these four viruses. The initial isolation of CVV was made from *Culiseta inornata* mosquitoes in Utah in 1956 and the virus, or subtypes of it, have since been detected across much of the United States as well as Canada, Mexico, Panama, Ecuador, and Jamaica.^{6–12} Cache Valley virus has been associated with two cases of severe human disease in the United States, the first of which occurred in North Carolina in 1995 and the second in Wisconsin in 2003.^{13,14} In addition, Fort Sherman virus, an antigenic subtype of CVV, was responsible for a human case of febrile illness in Panama in 1985.⁹

Cache Valley virus is also a pathogen of ungulates, and CVV infections in sheep are common and can result in embryonic and fetal death, stillbirths, and multiple congenital defects.^{15–18} This virus has also been isolated from a sick caribou and an apparently healthy horse and cow, and antibodies to this virus have been detected in a variety of other vertebrates including deer, elk, goats, and pigs.^{18–22} The seroprevalence for CVV in white-tailed deer in disease-endemic areas of the United States is often high and usually exceeds 70%.^{21,23,24} In this region, white-tailed deer have been implicated as the natural reservoir host of CVV.²¹

Sequence and phylogenetic data indicate that CHLV is most likely a natural reassortant that acquired its S RNA segment from CVV and its M and L RNA segments from Potosi virus (POTV).⁴ A single isolation of this virus has been made from a pool of *Ochlerotatus (Aedes) taeniorhynchus* collected in Merida in the Yucatan Peninsula in 2007.^{4,5} The natural reservoir host(s) of CHLV has not been determined, and it is not known whether this virus is a pathogen of humans or other vertebrates. Potosi virus, the M and L segment donor of CHLV, has been identified in several states in the eastern and central United States, including Texas, although it could also be present in Mexico because it is one of the precursor viruses of CHLV^{25–28} (Tesh R, Travassos da Rosa A, unpublished data). Potosi virus is not a recognized pathogen of humans or other vertebrates. The natural reservoir host of POTV is also suspected to be white-tailed deer.²¹

Kairi virus was originally isolated from mosquitoes in Trinidad in 1955, and later was isolated from mosquitoes and wild vertebrates in Brazil, mosquitoes in Colombia, and a febrile horse in Argentina.^{29–32} More recently, a single isolation of KRIV was made from a pool of *Oc. taeniorhynchus* collected in Merida in 2007.^{5,33} Antibodies to KRIV were detected in 5% of humans sampled in Argentina in 2004 and 2005.³⁴ In addition, antibodies that neutralized KRIV were identified in 48% of horses sampled in Argentina in 1983 and 1984.³⁵

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Two other members of the BUN serogroup known to be present in Latin America are Maguari virus (MAGV) and Wyeomyia virus (WYOV), although neither has been reported in the Yucatan Peninsula. Maguari virus has been isolated from mosquitoes, and antibodies to this virus have been detected in humans, horses, sheep, and cattle in various parts of South America and the Caribbean islands.^{36–38} Wyeomyia virus has been isolated from mosquitoes and a human, and antibodies to this virus have been detected in humans in Central America, South America, and the Caribbean islands.^{36,39}

As noted earlier, SOURV belongs to the CAL serogroup. This poorly characterized virus was originally isolated from mosquitoes in New Jersey in 1960⁴⁰ and later from mosquitoes in Pennsylvania,⁴¹ Georgia (Mead DG, unpublished data) and the Yucatan Peninsula.⁶ The SOURV isolate from Mexico is genetically and serologically distinct from the prototype strain and represents a novel subtype of SOURV.⁴² Until now, there have been no published studies that reported detection of antibodies to SORV in vertebrates. Consequently, the host range of this virus has not been defined. The International Committee on Taxonomy of Viruses has assigned the acronym of SORV to this virus, but we use SOURV in this article because the acronym SORV is also used for Sororoca virus, which was discovered first.

There is no recent information on the seroprevalence of orthobunyaviruses in vertebrates in the Yucatan Peninsula. Therefore, the overall goal of this study was to determine the seroprevalence of orthobunyaviruses in livestock in this region. To achieve this goal, an archived collection of serum samples from various species of domestic vertebrates were assayed by plaque reduction neutralization tests (PRNTs) using the four orthobunyaviruses (CVV, CHLV, KRIV, and SOURV) isolated during recent entomologic investigations in the Yucatan Peninsula, as well as two other orthobunyaviruses (MAGV and WYOV) known to be present in Central and South America.

MATERIALS AND METHODS

Description of study sites. Domestic animals were sampled in 26 study sites located in 5 municipalities (Figure 1). Four municipalities (Panaba, Tizimin, Merida, and Tzucacab) are in Yucatan State and one (Jose Maria Morelos) is in Quintana Roo State. Yucatan and Quintana Roo are two of the three states that comprise the Yucatan Peninsula of Mexico. All

FIGURE 1. Geographic locations of the five municipalities in the Yucatan Peninsula of Mexico from which livestock were sampled.

study sites were on privately owned ranches or farms. The climate and topography of the study sites are similar. The climate is tropical. The average annual rainfall in each study site ranges from 600 to 1,100 mm, the average annual temperature is 26°C, and the average elevation is < 20 meters.

Sample population and serum collections. Blood samples were obtained from horses (n = 182), sheep (n = 31), chickens (n = 37), turkeys (n = 5) and a dog (n = 1) from September 2007 through October 2008. The horses were from the municipalities of Panaba (n = 108), Tizimin (n = 63), and Jose Maria Morelos (n = 11). The sheep, dog, and chickens were from Merida, and the turkeys were from Tzucacab. According to the owners, none of the animals had ever been outside the Yucatan Peninsula. All animals were regularly monitored (usually daily) by their caregivers for signs of illness. Six horses had clinical signs at the time of sampling (fever, ataxia, lethargy, depression, paralysis, and/or encephalitis); all other animals appeared healthy. The age range of the horses was 8 months to 15 years, and the mean age was 6.7 years. The age range of the sheep was 4 months to 6 years, and the mean age was 17 months. Ages of the dog, chickens, and turkeys were not recorded.

Plaque reduction neutralization tests. The PRNTs were conducted according to standard methods⁴³ using CHLV (strain CHLV-Mex07), CVV (strain CVV-478), KRIV (strain KRIV-Mex07), SOURV (strains SORV-252 and NJO-94f), MAGV (strain BeAr7272) and WYOV (strain prototype). South River virus (strain NJO-94f), MAGV, and WYOV were obtained from the World Arbovirus Reference Collection at the University of Texas Medical Branch in Galveston, Texas. The SOURV strain NJO-94f was originally isolated from mosquitoes in New Jersey in 1960.⁴⁰ The MAGV strain BrAr7272 was obtained from mosquitoes in Brazil in 1957.²⁹ The WYOV prototype was originally isolated from mosquitoes in Colombia in 1940.⁴⁴ All other viruses were isolated during our previous entomologic investigations in the Yucatan Peninsula and have been described elsewhere.^{4–6}

The PRNTs were performed using African green monkey kidney (Vero) cells. Initially, all serum samples were screened at a single dilution of 1:20. Serum samples that tested positive for antibodies to any of these viruses were further diluted and tested by PRNT to determine their end-point titers. Titers were expressed as the reciprocal of highest serum dilutions yielding $\geq 90\%$ reduction in the number of plaques (PRNT₉₀). For etiologic diagnosis, the PRNT₉₀ antibody titer to the respective virus was required to be at least four-fold greater than that to the other viruses tested. The exception to this rule was when the PRNT₉₀ titers for two or more virus species were $\geq 1,280$. In such instances, the animal was suspected to have had at least two orthobunyavirus infections but was assigned the conservative diagnosis of seropositive to an undetermined orthobunyavirus(es) to avoid potential misdiagnosis because antibody responses in vertebrates sequentially infected with orthobunyaviruses are not well understood. There is only one report that describes the antibody responses in vertebrates experimentally inoculated with two different orthobunyaviruses²¹ and, to the best of our knowledge, high PRNT titers have not been reported in vertebrates in Mexico.

Complement fixation tests. Complement fixation (CF) tests were performed to determine whether this technique can differentiate between antibodies to CHLV and POTV. The PRNT cannot be used for such purposes because these

two viruses share the same M RNA segment and therefore their surface glycoproteins are antigenically indistinguishable. However, complement-fixing antigenic determinants are associated with the S segment–encoded nucleocapsid protein. The CF tests were performed using a microtiter technique with two full units of guinea pig complement.⁴³ Titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0 to 4+. Viral antigens for the CF test were prepared from newborn mouse brains that had been inoculated with CHLV (strain CHLV-Mex07) or POTV (89-3380). We also included CVV (strain Holden) in these experiments. Immune serum samples for the CF test were prepared in adult mice that had been inoculated with 10% suspensions of infected suckling mouse brain of CHLV, CVV, or POTV. The immunization schedule consisted of four intraperitoneal injections of suspensions mixed with the complete Freund's adjuvant given at weekly intervals.

Isolation of RNA and reverse transcription polymerase chain reactions. Total RNA was extracted from serum samples of all symptomatic livestock using the QIAamp viral RNA extraction kit (QIAGEN, Valencia, CA) and analyzed by reverse transcription polymerase chain reaction using orthobunyavirus-reactive and CHLV-reactive primers. The orthobunyavirus-specific primers, BCS82 (5'-ATG ACT GAG TTG GAG TTT CAT GAT GT-3') and BCS332V (5'-TGT TCC TGT TGC CAG GAA AAT-3'), are specific for a 251-nucleotide region of the S RNA segment.⁴⁵ The CHLV-reactive primers, CHLV-M1488-F (5'-TGA TAC TGG CAG CAG CAG AGA CAG-3') and CHLV-M1870-R (5'-GGC TGT TAG AAT GCC TTG CAC ATG-3'), are specific for a 387-nucleotide region of the M RNA segment. Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), and PCRs were performed using *Taq* polymerase (Invitrogen) according to the manufacturer's instructions.

RESULTS

Seroprevalence of orthobunyaviruses in horses. Antibodies to one or more orthobunyaviruses were detected by PRNT in serum samples from 169 (92.9%) of 182 horses. Of these horses, 60 (33.0%) were seropositive for CHLV, 48 (26.4%) were seropositive for CVV, 1 (0.5%) was seropositive for SOURV, and 60 (33.0%) had antibodies to an undetermined orthobunyavirus (Table 1). The CHLV-seropositive horses had CHLV PRNT₉₀ titers of 40 (n = 1), 80 (n = 2), 160 (n = 5), 320 (n = 6), 640 (n = 17), 1,280 (n = 12), 2,560 (n = 15), and 5,120 (n = 2). The CVV-seropositive horses had CVV PRNT₉₀ titers of 80 (n = 1), 160 (n = 3), 320 (n = 12), 640 (n = 19), 1,280 (n = 10), 2,560 (n = 2), and 5,120 (n = 1). The SOURV-seropositive horse had a PRNT₉₀ titer of 320 when

NJO-94f (the SOURV isolate from New Jersey) was used (H-133 in Table 2). Interestingly, the PRNT₉₀ titer for this horse was eight-fold lower when the SOURV isolate from Mexico was used in the PRNT analysis. Five of the 60 horses seropositive for an undetermined orthobunyavirus(es) had PRNT₉₀ titers $\geq 1,280$ for at least two orthobunyaviruses (CHLV, CVV, and/or KRIV). Of the remaining 55 horses with antibodies to an undetermined orthobunyavirus(es), the PRNT₉₀ titer was usually highest when CHLV or CVV was used in the PRNT analysis and often there was a two-fold difference between the highest and second highest titer. Representative PRNT data from 12 horses with antibodies to orthobunyaviruses are shown in Table 2.

The seroprevalence of orthobunyaviruses in horses in all three municipalities was high. Antibodies to orthobunyaviruses were detected in 58 (92.1%) of 63 horses in Tizimin, 100 (92.6%) of 108 horses in Panaba, and 11 (100%) of 11 horses in Jose Maria Morelos. Of the 63 horses sampled in Tizimin, 22 (34.9%) were seropositive for CHLV, 14 (22.2%) were seropositive for CVV, 21 (33.3%) had antibodies to an undetermined orthobunyavirus, 1 (1.6%) was seropositive for SOURV, and 5 (7.9%) were negative for orthobunyavirus-specific antibody. Of the 108 horses sampled in Panaba, 33 (30.6%) were seropositive for CHLV, 32 (29.6%) were seropositive for CVV, 35 (32.4%) had antibodies to an undetermined orthobunyavirus, and 8 (7.4%) were negative for orthobunyavirus-specific antibody. Of the 11 horses sampled in Jose Maria Morelos, 5 (45.5%) were seropositive for CHLV, 2 (18.2%) were seropositive for CVV, and 4 (36.4%) had antibodies to an undetermined orthobunyavirus.

The age range of the 169 horses with orthobunyavirus-specific antibody was 8 months to 15 years, and the mean age was 7.0 years. The age range of the 13 horses negative for orthobunyavirus-specific antibody was 12 months to 6 years, and the mean age was 2.4 years. Six horses had clinical signs at the time of sampling. Of these horses, two were seropositive for CHLV, three were seropositive for an undetermined orthobunyavirus, and one was negative for orthobunyavirus-specific antibody. Viral RNA was not detected in the serum of any symptomatic horses by reverse transcription polymerase chain reaction using orthobunyavirus or CHLV-specific primers.

Seroprevalence of orthobunyaviruses in sheep. Antibodies to orthobunyaviruses were detected by PRNT in serum samples from 29 (93.5%) of 31 sheep. Of these sheep, 6 (19.3%) were seropositive for CHLV, 3 (9.7%) were seropositive for CVV, 4 (12.9%) were seropositive for SOURV, 16 (51.6%) had antibodies to an undetermined orthobunyavirus, and 2 (6.5%) were negative for orthobunyavirus-specific antibodies (Table 1). The CHLV-seropositive sheep had CHLV PRNT₉₀ titers of 80 (n = 1), 160 (n = 1), 1,280 (n = 2),

TABLE 1
Seroprevalence of orthobunyavirus neutralizing antibodies in livestock in Yucatan Peninsula of Mexico*

Vertebrate	No. (%) of livestock seropositive for each orthobunyavirus						
	CHLV	CVV	SOURV	KRIV	MAGV	WYOV	Undetermined†
Horse	60/182 (33.0)	48/182 (26.4)	1/182 (0.5)	0/182 (0)	0/182 (0)	0/182 (0)	60/182 (33.0)
Sheep	6/31 (19.3)	3/31 (9.7)	4/31 (12.9)	0/31 (0)	0/31 (0)	0/31 (0)	16/31 (51.6)
Dog	0/1 (0)	0/1 (0)	1/1 (100)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
Chicken	0/37 (0)	0/37 (0)	0/37 (0)	0/37 (0)	0/37 (0)	0/37 (0)	4/37 (10.8)
Turkey	1/5 (20.0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

* CHLV = Cholov virus; CVV = Cache Valley virus; SOURV = South River virus; KRIV = Kairi virus; MAGV = Maguari virus; WYOV = Wyeomyia virus.

† Undetermined orthobunyavirus (see text).

TABLE 2
Plaque reduction neutralization test results for a subset of livestock with antibodies to orthobunyaviruses, Yucatan Peninsula of Mexico*

Sample ID†	PRNT ₉₀ titer							PRNT diagnosis
	CHLV	CVV	KRIV	SOURV‡		MAGV	WYOV	
				NJO-94f	SORV-252			
H-006	320	40	80	–	–	–	–	CHLV
H-010	–	20	–	–	–	–	–	Undetermined
H-021	40	160	20	–	–	40	–	CVV
H-037	40	80	20	–	–	–	–	Undetermined
H-057	160	40	40	–	–	–	–	CHLV
H-067	20	640	20	–	–	160	–	CVV
H-133	–	–	–	320	40	–	–	SOURV
H-139	5,120	1,280	1,280	–	20	80	–	Undetermined
H-161	2,560	160	320	20	40	20	–	CHLV
H-165	320	1,280	80	–	–	160	–	CVV
H-193	320	80	160	–	20	–	–	Undetermined
H-263	10,240	40	2,560	–	–	20	–	Undetermined
S-234	160	–	20	–	–	–	–	CHLV
S-236	–	–	–	20	–	–	–	Undetermined
S-239	40	–	–	20	–	–	–	Undetermined
S-240	20	1,280	–	–	–	–	–	CVV
S-243	160	–	–	640	80	–	–	SOURV
S-245	10,240	640	2,560	320	160	80	–	Undetermined
S-252	1,280	80	160	20	160	40	–	CHLV
S-262	2,560	640	640	320	–	640	–	CHLV
D-194	20	–	–	160	–	–	–	SOURV
C-223	–	–	20	–	–	–	–	Undetermined
C-225	–	–	20	–	–	–	–	Undetermined
T-005	80	–	20	–	–	–	–	CHLV

* PRNT = plaque reduction neutralization test; CHLV = Cholul virus; CVV = Cache Valley virus; SOURV = South River virus; KRIV = Kairi virus; MAGV = Maguari virus; WYOV = Wyeomyia virus; – = < 20.

† Sample IDs beginning with a H, S, D, C, and T denote serum samples from horses, sheep, dogs, chickens, and turkeys, respectively.

‡ Two SOURV isolates (NJO-94f and SORV-252) were used in the PRNT analysis.

2,560 (n = 1), and 5,120 (n = 1). The CVV-seropositive sheep had CVV PRNT₉₀ titers of 40 (n = 1), 160 (n = 1), and 1,280 (n = 1). The SOURV-seropositive sheep all had SOURV PRNT₉₀ titers of 640 when strain NJO-94f was used. In contrast, the PRNT₉₀ titers for these four sheep ranged from 20 to 80 when SORV-252 was used. Of the 16 sheep seropositive for an undetermined orthobunyavirus(es), the PRNT₉₀ titer was usually highest when CHLV was used for the PRNT analysis and often there was a two-fold difference between the highest and second highest titer. The two sheep negative for orthobunyavirus-specific antibody were five and seven months of age, and the mean age of the sheep seropositive for orthobunyaviruses was 17.7 months. Representative PRNT data from eight sheep with antibodies to orthobunyaviruses are shown in Table 2.

Seroprevalence of orthobunyaviruses in other vertebrates. The single dog was seropositive for SOURV (Table 1). As observed with the horses and sheep, the highest SOURV titer occurred when the PRNTs were performed with NJO-94f (D-194 in Table 2). Four (10.8%) of the 37 chickens had antibodies to an undetermined orthobunyavirus(es). All four of these chickens had KRIV PRNT₉₀ titers of 20, and the titers for all other viruses tested were < 20. Antibodies to CHLV

were detected in 1 (20.0%) of the 5 turkeys. The CHLV PRNT₉₀ titer for this bird was 80 (T-005 in Table 2).

Complement fixation tests. The CF tests were performed using antisera and antigens from mice that had been experimentally inoculated with CHLV, CVV, or POTV. All antisera gave indistinguishable titers (less than a four-fold change in both directions), indicating that this test cannot be used to differentiate between antibodies to these three viruses (Table 3). Therefore, this technique was not used to further analyze serum samples from livestock in the Yucatan Peninsula.

DISCUSSION

We provide serologic evidence that orthobunyaviruses commonly infect livestock in the Yucatan Peninsula. Antibodies to orthobunyaviruses were identified in all five vertebrate species examined and orthobunyavirus activity was detected in all five municipalities represented in this study. The seroprevalence for orthobunyaviruses in horses and sheep was particularly high (approximately 93%) and at least three viruses (CHLV, CVV, and SOURV) were shown to be responsible for these infections. There is no other published information on the host range of the recently described CHLV or the poorly characterized SOURV, nor are there data on the seroprevalence of these two viruses in vertebrates in other geographic regions. A number of serologic investigations have determined the seroprevalence of CVV in various vertebrates but these studies have mostly been confined to the United States.^{21–23,37,46–51} For instance, 19% of sheep sampled in Texas in 1981 were seropositive for CVV.⁴⁶ In another study, antibodies that neutralized CVV were detected in 84 (95%) of 88 horses, 61 (52%) of 118 cattle, and 6 (27%) of

TABLE 3
Results of complement fixation tests performed with Cholul, Potosi and Cache Valley viruses*

Antigens	Immune serum (antibodies)		
	Cholul virus	Potosi virus	Cache Valley virus
Cholul virus	≥ 1,024	≥ 1,024	256
Potosi virus	≥ 1,024	≥ 1,024	256
Cache Valley virus	≥ 1,024	512	256

* Values refer to the highest dilution of antiserum with complement fixation activity.

22 sheep in Virginia and Maryland during 1957–1961.²² Neutralizing antibodies to CVV were also detected in 100 (72%) of 138 white-tailed deer in Minnesota in 1988 and 1989.²³ Therefore, the moderately high seroprevalence for CVV in sheep and horses (10% and 27%, respectively) and the high overall seroprevalence for orthobunyaviruses in these vertebrate species (approximately 93%) in the Yucatan Peninsula are not dissimilar to the seroprevalences reported for ungulates sampled in other serologic investigations. However, one important aspect of this study is that it provides recent information on the seroprevalence of orthobunyaviruses in vertebrates in Mexico.

Cholul virus and POTV share the same M RNA segment. Therefore, because their surface glycoproteins are antigenically indistinguishable, antibodies to these proteins cannot be differentiated by PRNT. Although complement-fixing antigenic determinants are associated with the S segment-encoded nucleocapsid protein, the CF test was also unable to differentiate between antibodies to CHLV and POTV. Thus, we cannot dismiss the possibility that POTV was the cause of infection in some or all of the 67 animals (60 horses, 6 sheep, and 1 turkey) considered to be seropositive for CHLV. However, we consider it more likely that the aforementioned animals had been infected with CHLV because this virus has been isolated in the Yucatan Peninsula, and there is no direct evidence that POTV is present in this region. It is noteworthy that serum samples from the six sheep considered to be seropositive for CHLV were collected in Merida in 2008. The pool of mosquitoes yielding CHLV in our study was collected in Merida less than 12 months earlier.^{4,5} Nevertheless, we cannot dismiss the possibility that POTV was responsible for some or all of these infections and that a more accurate PRNT diagnosis for the aforementioned animals could be seropositive for CHLV or POTV or seropositive for CHLV or a CHLV-like virus.

Five horses and two sheep had PRNT₉₀ titers $\geq 1,280$ for at least two orthobunyaviruses. We believe that this is the first report of high PRNT titers in vertebrates in Mexico with naturally acquired orthobunyavirus infections. High antibody titers are often reported in flavivirus serologic investigations performed in geographic areas where multiple flaviviruses circulate and have been attributed to exposure to two or more flaviviruses.^{52–54} For example, all four dengue flaviviruses are present in Mexico and patients in this region often have high PRNT titers to all serotypes.⁵³ It seems likely that the aforementioned horses and sheep had infections with at least two orthobunyaviruses and that the responses we detected may have been anamnestic responses. In this regard, Blackmore and Grimstad reported high neutralizing antibody titers to CVV in white-tailed deer experimentally inoculated with CVV, then POTV.²¹ The mean $\pm$ SE reciprocal antibody titer for CVV by virus neutralization assay was 839 ± 228 at seven days post-inoculation with the secondary virus. This study is the only one to describe antibody responses in vertebrates experimentally inoculated with two orthobunyaviruses. Because the antibody responses in vertebrates with secondary orthobunyavirus infections are poorly understood as compared with secondary flavivirus infections, we have therefore interpreted our PRNT data with caution and have assigned the conservative diagnosis of seropositive for an undetermined orthobunyavirus(es) to avoid potential misdiagnosis. Nonetheless, it is feasible that these animals have been infected with two or more orthobunyaviruses and that secondary

orthobunyavirus infection could be a more accurate diagnosis. Alternatively, these animals may have produced unusually high neutralizing antibody titers after exposure to a single orthobunyavirus. If this is the case, these animals are seropositive for CHLV (three horses and two sheep), CVV (one horse), and an undetermined orthobunyavirus (one horse). For instance, horse H-139 has CHLV, CVV, and KRIV PRNT₉₀ titers of 5,120, 1,280 and 1,280, respectively (Table 2) and therefore could be considered seropositive for CHLV.

A high proportion of horses (33%) and sheep (52%) had antibodies to an undetermined orthobunyavirus(es). One explanation for this finding is that many of these animals had been exposed to two or more orthobunyaviruses, thus making it difficult to make a definitive diagnosis. If this hypothesis is correct, we consider it most likely that the orthobunyaviruses responsible for these dual infections are CHLV and CVV because these two viruses were the most common causes of infection in this study. However, in our studies, many animals seropositive for an undetermined orthobunyavirus did not have extremely high PRNT₉₀ titers. For example, the highest PRNT₉₀ titer for 41 of the 60 horses with antibodies to an undetermined orthobunyaviruses did not exceed 320. This observation does not necessarily refute our hypothesis. Blackmore and Grimstad reported data that imply that high neutralizing antibody titers are not always a consequence of sequential orthobunyavirus infections.²¹ Seven days after inoculation with the second virus, the mean $\pm$ SE reciprocal antibody titers in deer sequentially inoculated with POTV followed by CVV were 206 ± 60 and 96 ± 16 , respectively. It is also important to note the approximately two-fold difference in mean antibody titers in these deer because many of the horses and sheep with undetermined orthobunyavirus infections also exhibited a two-fold difference between their highest and second highest PRNT titer.

Another explanation for the high proportion of horses and sheep with antibodies to an undetermined orthobunyavirus(es) is that some of these animals had been infected with an orthobunyavirus not included in the PRNT analysis. However, our PRNTs were not restricted to orthobunyaviruses known to be present in the Yucatan Peninsula. Two additional orthobunyaviruses (MAGV and WYOV), which have been reported elsewhere in Latin America and can infect some of the animal species we studied were included. Nevertheless, a subset of animals may have been infected with another orthobunyavirus such as Northway, Tensaw, or Main Drain viruses. Although these viruses have not been reported in Mexico, they have been associated with livestock infections in the United States.^{47,55} For instance, antibodies to Northway virus were identified in 44% of horses sampled in California during 1968–1972.⁵⁵ The establishment of a continuous entomologic-based arbovirus surveillance program in the Yucatan Peninsula would enable identification of other orthobunyaviruses that may be present in this region.

Six horses had signs of illness at the time of sampling, including two horses (H-2 and H-265) that were seropositive for CHLV. Horse H-2 had neurologic signs (facial paralysis and encephalitis) and later died, and horse H-265 exhibited posterior ataxia. Three of the other symptomatic horses (H-116, H-263, and H-264) were seropositive for an undetermined orthobunyavirus. Horse H-116 exhibited lethargy, horse H-263 had a fever and posterior ataxia and later died, and horse H-264 had posterior ataxia. The remaining horse was negative

for orthobunyavirus-specific antibody. It was not known whether the clinical signs in horses H-2 and H-265 were a result of CHLV infection, but we speculate that this was not the case because CVV and POTV, the two precursor viruses of CHLV, are not recognized equine pathogens. An IgM enzyme-linked immunosorbent assay has not been developed for any BUN serogroup virus, such an assay would be a significant advance in orthobunyavirus surveillance studies because it would enable detection of acute infections. The PRNTs can be used to identify recent orthobunyavirus infections when paired acute-phase and convalescent-phase serum samples are available, but for our studies only single serum samples were available from each animal.

Antibodies to CHLV and an undetermined KRIV-like virus were identified in 1 (20%) of 5 turkeys and 4 (10.8%) of 37 chickens, respectively. Several other studies also describe the identification of antibodies to BUN serogroup viruses in birds.^{56–58} For instance, antibodies that neutralized MAGV were detected in 69 (10.6%) of 649 free-ranging birds of various species in Argentina in 2004 and 2005.⁵⁶ Our PRNT data indicate that the seroprevalence for orthobunyaviruses in domestic birds is much lower compared with seroprevalences in mammals in the Yucatan Peninsula. One explanation for this finding is that the major vectors of orthobunyaviruses in this region have a preference for mammalian blood. In this regard, all the orthobunyaviruses isolated in our recent entomologic investigations in the Yucatan Peninsula were obtained from *Oc. taeniorhynchus*.^{5,6} Although the host-feeding preference of *Oc. taeniorhynchus* in the Yucatan Peninsula has not been determined, *Oc. taeniorhynchus* in other regions of North America have been shown to feed almost exclusively on large mammals.^{59–61}

Antibodies to SOURV were identified in 4 (12.9%) of 31 sheep, 1 (0.5%) of 182 horses, and the single dog sampled in this study. Surprisingly, the PRNT₉₀ titers of these animals were always greater when the PRNTs were performed with NJO-94f, the SOURV isolate collected in New Jersey in 1960, compared with SORV-252, which was isolated from mosquitoes in the Yucatan Peninsula in 2008.^{6,40} The NJO-94f PRNT₉₀ titers were usually 8–16-fold greater than their corresponding SORV-252 PRNT₉₀ titers and this finding is also surprising because such differences are more indicative of distinct viral species than subtypes. We recently demonstrated by cross-PRNT using serum samples from mice inoculated with SORV-252 and NJO-94f, and these isolates are distinct subtypes of SOURV.⁴² The contrasting serologic findings between the two studies could be caused by differences in the antibody responses of rodents and larger mammals after SOURV infection or to the duration of time between virus infection and serum collection. Although unlikely, the consistently higher NJO-94f PRNT₉₀ titers compared with the corresponding SORV-252 PRNT₉₀ titers could be the result of the NJO-94f subtype also circulating in the Yucatan Peninsula, although it has not yet been found there.

In summary, we provide serologic evidence that orthobunyaviruses commonly infect livestock in the Yucatan Peninsula. It is not known whether these viruses are also responsible for morbidity and mortality in livestock in this region, but future research is necessary to address this issue. The high seroprevalence for orthobunyaviruses in mammals also implies that the major orthobunyavirus vectors in the Yucatan Peninsula have a strong preference for mammalian

blood. It is therefore important that the potential impact of orthobunyaviruses on human health in the Yucatan Peninsula be determined. This is especially true for CVV because this virus is a recognized pathogen of humans.

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