Prevalence of Infection with Hantavirus in Rodent Populations of Central Argentina

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We studied hantavirus seroprevalence and virus variability in rodent populations in Diego Gaynor, northwest of Buenos Aires province, Argentina. Rodent samplings were conducted in railroads and cropfield borders in March and July 1999, September and December 2000, and March 2001. Antibody detection was performed by an enzyme link immunosorbent assay (ELISA), using the recombinant nucleoprotein of Andes (AND) virus as antigen. Tissue samples were taken from positive antibody individuals in order to confirm the presence of hantavirus genomic material and to identify virus genotypes. Akodon azarae *was the most abundant species, followed by* Oligoryzomys flavescens*, while* Calomys laucha *and* C. musculinus *were rarely caught. We found a rate of seroprevalence of 9.3% for a total sample of 291* A. azarae *and 13.5% for 37* O. flavescens*. After molecular analyses of hantavirus, we confirmed the presence of hantavirus genomic material in 16 individuals with ELISA (+) results and two individuals with ELISA (-). Four amplimers for each species were sequenced and compared to the corresponding sequences of representative hantaviruses. We identified the AND Cent Lec from three* O. flavescens*, and the Pergamino virus from four* A. azarae *and from one* O. flavescens*.* A. azarae *males had higher seroprevalence than females, and heavier individuals showed higher seroprevalence than lighter ones. We did not find seroprevalence differences according to sex in* O. flavescens*, although this result may have been produced by the low sample size. The lowest seroprevalence was found in a period of high rodent density, when juveniles prevailed in the population. We found higher seroprevalences than those detected in previous studies for other localities of central Argentina where cases of hantavirus pulmonary syndrome (HPS) have been reported. The presence of AND Cent Lec virus in rodent populations of the study area, which is responsible of HPS cases in central Argentina, suggests that human populations are at risk of HPS disease, although there were not reported cases of this disease until today.*

Key words: sigmodontine rodents - hantavirus pulmonary syndrome - seroprevalence - Argentina

Hantavirus pulmonary syndrome (HPS) is a severe and often fatal cardiopulmonary disease that affects many people in America. After the outbreak of HPS in 1993 in Southwestern United States (Nichol et al. 1993), there was an increase in reports from cases in South America. Between 1993-2001, there have been 310 confirmed cases of HPS in Argentina, 91 in Paraguay, 204 in Chile, 167 in Brazil, 27 in Uruguay and 11 in Bolivia (PAHO 2002).

Rodent species of the subfamily Sigmodontinae are the main reservoirs of different types of hantaviruses throughout the continent. *Calomys laucha* was identified as the reservoir of the virus Laguna Negra, the etiologic agent of HPS in Paraguay (Yahnke et al. 2001), while three species of *Oligoryzomys* have been related to hantavirus transmission: *O. longicaudatus* (in Chile and Southern Argentina), *O. chacoensis* (in Northern Argentina, Padula et al. 2002), and *O. flavescens* in Central Argentina and Uruguay (Levis et al. 1998, González della Valle et al. 2002).

Serosurveys of wild rodents conducted in areas of Brazil where cases of HPS occurred found that *Bolomys lasiurus* and *Oligoryzomys* spp. may be potential reservoirs of hantaviruses in this country (Romano-Lieber et al. 2001). On the other hand, another study conducted in Brazil found a a seroprevalence of 28.8% in *Holochilus sciureus* (Vasconcelos et al. 2001).

In Argentina, the first cases of HPS were recorded and characterized in 1995 in the Southwest (López et al. 1996). Nowadays, the occurrence of cases is concentrated in three geographically isolated areas: North (Salta and Jujuy provinces), Center (Buenos Aires, Santa Fe and Entre Ríos provinces), and Southwest (Neuquén and Río Negro provinces). In an extensive study conducted in sites were HPS cases occurred, many sigmodontine species were found infected with hantavirus, with seroprevalences which varied between 1.5 and 10.2%, depending on the area and species (Calderón et al. 1999, Cantoni et al. 2001, Sosa Estani et al. 2002, Gonzalez della Valle et al. 2002). López et al. (1996, 1997) described the virus associated to HPS cases in Argentina, Andes virus (AND), as a new type of hantavirus. This virus circulates in Argentina, Chile and Uruguay with different lineages that are

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characteristics of the different areas (Padula et al. 2000a). Although three species of the genus *Oligoryzomys* were identified as associated to hantavirus that caused HPS in Argentina, the reservoirs of two lineages of virus which caused HPS in Buenos Aires (AND Cent Buenos Aires and AND Cent Plata; Martínez et al. 2001) have not been identified yet. Moreover, there are two other types of virus, which have not been associated to human disease, until now, were identified from other sigmodontine species inhabiting the Buenos Aires province: Maciel virus (in *Necromys obscurus*), and Pergamino (in *Akodon azarae*) (Levis et al. 1998).

Previous studies of rodent hantavirus reservoirs in US (Abbott et al. 1999, Mills et al. 1999) and Argentina (Cantoni et al. 2001) suggest that the prevalence of infection is higher for adult males than for females and juveniles. Differences among demographic groups have been related to behavioral factors as agonistic interactions, to home range size, and to the age effect that increase the probability of being infected, suggesting that horizontal transmission is the main mechanism of maintaining the virus in nature (Abbott et al. 1999, Mills et al. 1999). Although seroprevalence variations according to changes in population density and structure have been described (Graham & Chomel 1997, Kuenzi et al. 1999, Mills et al. 1999, Biggs et al. 2000, Cantoni et al. 2001), there is still not a clear pattern of variation of seroprevalence with density. When higher density is accompanied with an increase in the proportion of juveniles in the population, a lower seroprevalence have been related to a dilution effect (Mills et al. 1999).

This work shows results of a study conducted in order to study the prevalence of infection with different types of hantavirus in rodent populations of northwest Buenos Aires province, Argentina, and its variation according to species and demographic sub classes.

MATERIALS AND METHODS

Study area - Fieldwork was conducted at Diego Gaynor (34º 8'S, 59º14'W), Buenos Aires Province, Argentina (Fig. 1). The small rodent community in this area is composed by the sigmodontines *A. azarae*, *C. laucha*, *C. musculinus*, *O. flavescens*, *Oxymycterus rufus*, the cavid *Cavia aperea* and the murines *Rattus norvegicus*, *R. rattus* and *Mus musculus*.

Rodent populations show an annual cycle of population density in the study area, with a minimum in spring and a peak in late autumn-winter. The reproductive period lasts between September-October (spring) to autumn (March-April). The population structure changes along the seasons: in spring there is a great proportion of over- wintering adults, along the reproductive period are recruited juveniles and in autumn the population is composed by many age classes. Populations turnover annually, and the ecological longevity is ≤ 6 months (Zuleta et al. 1988).

The original vegetation consisted of matted grasses 1 m high and trees were absent. Nowadays, the native vegetation is replaced by areas intensely cultivated (the most common crops are wheat, maize, sunflower and soybean), natural pastures, poultry farms and fields with cattle. Most of the remaining native plants are restricted to linear and less disturbed habitats including cropfield edges (borders), fence rows, roadsides and railroads (Mills et al. 1991, Busch & Kravetz 1992).

Buenos Aires province is subdivided in political subunits, called partidos or departments, which have local political and sanitary authorities. Up to now, there were not reported human cases of HPS in the department of Exaltación de la Cruz (to which the study area belongs), but there were cases in the neighboring departments of Zárate, Campana and Pilar (Fig.1).

Rodent sampling and virologic analyses - Trapping was conducted in cropfield borders and railroads. We conducted five rodent samplings between March 1999 and March 2001 (Table). In March and July 1999 we conducted only removal samplings, while in the other dates we performed both capture-mark-recapture and removal samplings. The total trapping effort was of 750 trap-nights in each sampling. We used Sherman live traps which were active for three consecutive nights. The distance between neighbor traps was 10 m. Captured

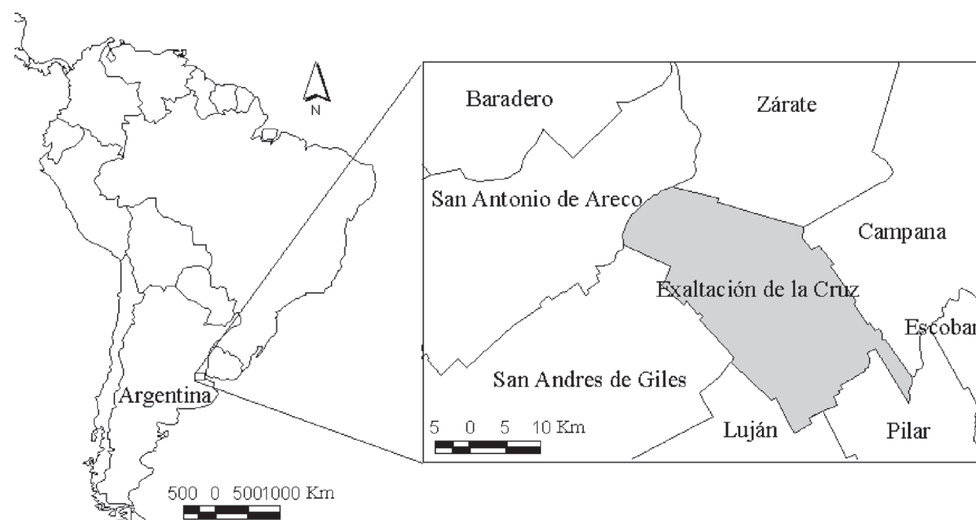

Fig. 1: location of the study area

animals were collected each morning and carried to a centrally located field processing station. Animals were anesthetized with chloroform or ether. For each animal captured we recorded the species (according to external morphology), date and localization of capture, corporal length, weight, sex and reproductive condition. Reproductively active females (pregnant, with evidence of lactation or with open vaginas), and active males (with scrotal testes) were distinguished from reproductively inactive (females with closed vaginas and males with abdominal testes). We examined each animal for wounds (ear nicks, torn ears, scarred tails), and for evidences of infection by botfly larvae (Cuterebra, Diptera, Insecta), which may enhance the probability of infection (Mills & Childs 1998).

Blood samples were collected from retroorbital sinus using heparinized capillary tubes, following Mills et al. (1995) or from the tail of all individuals captured, and an enzyme-linked immunosorbent assay (ELISA) was performed, with the recombinant nucleoprotein of the virus AND as antigen, for detection of specific IgG antibodies in serum or whole-blood (Padula et al. 2000b). Rodents from capture-mark-recapture areas were released at the site of capture after given an individual mark. Rodents captured in removal areas which were positive in the ELISA test were euthanized for taking organ tissue samples from lung and kidney, following Mills et al. (1995). We conducted molecular analyses in order to confirm the presence of genomic hantavirus material for 18 seropositive animals from which we had organ samples, and from two negative *O. flavescens* which were found dead in traps. Total RNA was extracted from lung or kidney tissues using the guanidine isothiocyanate-acid phenol extraction procedure as described in Padula et al. (2000b). We used the RNAid kit (Bio 101) for RNA purification. Partial S and M segments were amplified by RT-PCR followed by a second round of nested or heminested PCR. We used specific oligonucleotide primers based on conserved regions of AND virus genome. Amplifications were conducted from nucleotides 69 to 324 from the nucleoprotein coding region of the S segment and from nucleotides 2 716 to 2 943 from the M segment. Positions of the S and M segment fragments were numbered relative to AND virus. The amplification products were purified and sequenced by an ABI 377 sequencer by the fluorescent technique (d Rhodamine Terminator Cycle Sequencing kit Applied Biosystem). The analysis and comparison of the nucleotide and aminoacid sequences was made by the CLUSTAL program, included in PC/GENE 6.8, Intelligenetics Inc., Mountain View, CA.

Data analysis - Rodent density was estimated as the number of different individuals captured in each 3 day trapping period. Seroprevalence was estimated as the number of rodents which were positive in the ELISA test divided by the number of different rodents captured and tested.

We compared the proportions of seropositive individuals according to sampling period, species, sex and reproductive condition by means of a Chi-square analysis when sampling sizes were sufficient to statistical comparisons. For *O. flavescens* we compared the sex ratio of infected animals by means of a binomial test, considering that the proportion of each sex in the infected sample must be equal to the proportion in the total sample. We separated *A. azarae* individuals into weight classes I: ≤ 20 g (juveniles and subadults), II: 21-30 g (young adults), III > 30 g (old adults). For those animals which were recaptured we considered their serocondition (positive or negative) only at the moment of first capture.

RESULTS

We captured a total of 335 rodents, six of them were recaptured once, totalizing a total of 341 blood samples. *A. azarae* was the most common species (86.9% of total individuals captured), followed by *O. flavescens* (11%). *C. laucha* and *C. musculinus* were rarely captured (2.1%). Only 4 (1.4%, n = 291) *A. azarae* had wounds and 12 (4.1%, n = 291) were infected with cuterebrids. We did not detect wounds or cuterebrids in any *O. flavescens* (n = 37).

Rodent abundance and population structure in the different sampling periods - The abundance of *A. azarae* varied between the two years studied, with a minimum in March 1999 and a maximum in March 2001 (Table). The pattern of variation among months within each year agreed with that observed for this species in other works, with increasing density from spring to autumn-winter (Zuleta et al. 1988).

In March 1999 the population was mainly formed by individuals of weight class III, while there was a low proportion of juveniles (class I). In July 1999 we found a higher proportion of class I, while there were few individuals of class III. In December 2000, there was a great proportion of the population in the first weight class, but all weight classes were represented. In March 2001 classes I and II were dominant in the population, while there were few individuals of class III (Fig. 2).

O. flavescens was less abundant than *A. azarae*, and it was more abundant in 1999 than in 2000-2001 (Table).

Antibody prevalence by species, sex and reproductive condition - AND virus antibody-reactive rodents were detected in the two most common captured species. Hantavirus seroprevalence was of 13.5% for a total sample of 37 *O. flavescens* and 9.3% for 291 *A. azarae.*

We did not find differences in seroprevalence between male and female *O. flavescens* (10%, n = 20, and 17.7%, n = 17, respectively, P = 0.28, binomial test).

A. azarae males were more frequently infected than females (15.3%, n = 157 and 2.3%, n = 133 for males and females, respectively; χ^2 = 14,48; df = 1, P < 0.001). Seroprevalence was higher for reproductively active *A. azarae* males (21.1%, n = 76) than for inactive ones (9.9%, n = 81, χ^2 = 3,78, df = 1, P = 0.05). Active females (n = 64) showed a seroprevalence of 4.7%, while there were not positive inactive females (n = 65), but we did not test for statistical differences because of the low sample size of positive females. Larger *A. azarae* (weight class III) showed a higher frequency of antibodies (40.6%, n = 32), than the other classes (I and II, χ^2 = 20.63, df = 2, P < .001).

We did not detect hantavirus antibodies in none of the seven *Calomys* analyzed.

Seroprevalence variations according to the sampling period - The few captures of *O. flavescens* in each sampling period prevented us to analyze differences in the proportion of positive animals among them.

TABLE

Hantavirus antibody seroprevalence and rodent abundance variations according to the sampling period

		Captured (Not recaptured)			Positive ELISA test			Positive
		Male	Female	Total	Male	Female	Total	PCR
March 1999	*Akodon azarae*	20	17	37	5	2	7	6
	Oligoryzomys flavescens	4	4	8	0	0	0	-
	Calomys sp.	1	3	4	0	0	0	-
July 1999	*Akodon azarae*	29	21	50	6	0	6	5
	Oligoryzomys flavescens	7	7	14	0	1	1	1
	Calomys sp.	0	0	0	-	-	-	-
September 2000	*Akodon azarae*	25	19	44	6	0	6	1
	Oligoryzomys flavescens	7	6	13	1	2	3	2 [a]
	Calomys sp.	1	1	2	0	0	0	-
December 2000	*Akodon azarae*	37	31	68	5	1	6	-
	Oligoryzomys flavescens	1	0	1	1	-	1	1
	Calomys sp.	0	0	0	-	-	-	-
March 2001	*Akodon azarae*	47	45	92	2	0	2	-
	Oligoryzomys flavescens	1	0	1	0	-	0	-
	Calomys sp.	0	1	1	-	0	0	-
Totals/ species	*Akodon azarae*	158	133	291	24	3	27	12
	Oligoryzomys flavescens	20	17	37	2	3	5	4 [a]
	Calomys sp.	2	5	7	0	0	0	

a: in September 2000 the polymerase chain reaction (PCR) was not conducted in one ELISA positive *O. flavescens* which was not removed.

Antibody seroprevalence in *A. azarae* was differentially distributed among samplings ($\chi^2 = 9.44$, df = 4, P = 0.05) with a minimum in March 2001 (3.1%, n = 87), when population density was high, and a maximum in March 1999 (18.9%, n = 37), when there was low density (Table).

Seroconversion rates of recapture animals - From six *A. azarae* recaptured only one time each (four males and two females), two males become positive during the three months period elapsed between captures (one between September and December 2000, and the other between December 2000 and March 2001), while the other two maintained their condition (one remained positive between September and December 2000 and the other negative between December 2000 and March 2001). Both females remained negative in both captures.

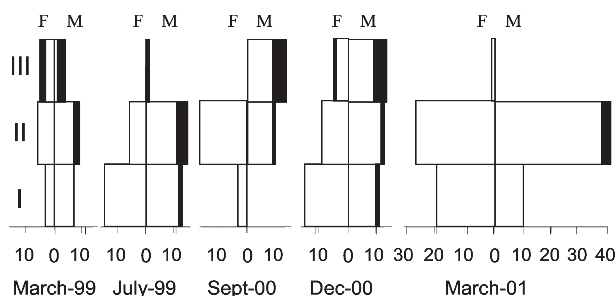

Fig. 2: distribution of mass classes (I, II, III) and seroprevalence in the *Akodon azarae* population in the different periods. In black: positive animals; F: female; M: male

Confirmation of presence of hantavirus genomic material and characterization of viral genotypes - In order to estimate hantavirus prevalence within the antibody positive population, total RNA extraction and partial viral genome amplification of the coding region from the S segment were performed when tissue samples were available. Molecular analyses was positive for the 4 ELISA positive of *O. flavescens*, and for 12 out of 14 *A. azarae* (Table). We preferred to study *O. flavescens* due to two reasons, one of them was the cost of the molecular analyses, and the other one was the previous result which confirmed the Pergamino genotype in *A. azarae* (Levis et al. 1998). Two additional antibody non reactive *O. flavescens* were positive for viral detection.

In order to characterize virus genotypes, four randomly selected amplimers from positive *A. azarae* and four amplimers from *O. flavescens* were sequenced and analyzed for comparison with corresponding sequences of representative hantavirus. Pergamino virus was characterized from four seropositive *A. azarae* and one negative *O. flavescens*. The four viruses from the *A. azarae* had the same sequence in the N conserved fragment. Pergamino virus from the *O. flavescens* differed in one out of 274 nucleotides in this fragment. AND Cent Lec was identified in two antibody positive and one antibody negative *O. flavescens*. None of the virus lineages circulating in central Argentina region studied previously (Padula et al. 2000a) differed from each other by more than 5% in the N conserved fragment. For this reason another fragment coding for the glycoprotein G2 from the M segment was sequenced in the three *O. flavescens* viruses. Sequence identity differences up to 17.2% and up to 2.7%

were seen in nucleotidic and aminoacidic levels, respectively, when the three G2 fragment sequences were compared.

DISCUSSION

Our results confirm *O. flavescens* as the reservoir of the AND Cent Lec virus, responsible of many HPS cases in central Argentina, and show that the virus is more extended in rodent populations than expected according to human cases. The identification of the Pergamino virus in *A. azarae* also agrees with previous studies (Calderón et al. 1999, Padula et al. 2000a). The detection of Pergamino virus in one individual of *O. flavescens* suggests that the virus was transmitted between *A. azarae* and *O. flavescens*, but it could had been an incidental infection which not resulted in persistent infection. However, this result also reveals that two different lineages of hantaviruses can be cocirculating in the same species at the same location.

The rates of hantavirus seroprevalence found in *A. azarae* and *O. flavescens* in this study were almost three times higher than those recorded by Calderón et al. (1999) in localities of Buenos Aires province where cases of HPS have been reported: 9.3, n = 291 versus 2.7%, n = 549 $P < 0.0001$ for *A. azarae* and 13.5, n = 37 versus 4.7%, n = 170 $P < 0.05$ for *O. flavescens,* for this study and Calderón's study, respectively, test of comparison of two percentages (Bailey 1981). Strong variations in seroprevalences are not only evident when comparing studies conducted by different authors, but also within habitats and times (Abbott et al. 1999, Mills et al. 1999). In Argentina and other areas of the Americas seroprevalence range between 3-14% (Calderón et al. 1999, Cantoni et al. 2001, Yahnke et al. 2001). However, the difference in seroprevalence could also be attributed to the different type of antigen used to estimate seroprevalence in each study (Sin Nombre and AND antigens for Calderón et al.'s and our study, respectively), although Toro et al. (1998) did not find differences in serologic results obtained with these two types of antigen.

The higher rates of hantavirus seroprevalence found in larger and reproductively active (i.e., older) individuals of *A. azarae*, and specially in males, is in agreement with that observed in *O. longicaudatus* in Southern Argentina by Cantoni et al. (2001), in *C. laucha* in Paraguay by Yahnke et al. (2001) in species of *Peromyscus* in the United States (Abbott et al. 1999, Kuenzi et al. 1999, Mills et al. 1999), and in *C. musculinus* infected with Junín virus (Mills et al. 1994). Differences in seroprevalence among age classes and sexes have been related to horizontal transmission that involves a higher chance of older individuals of being infected, since they have a longer period of exposition to the virus (i.e., the "age factor"). On the other hand, the higher prevalence of antibodies in *A. azarae* active males is probably related to their large range of movements (Cittadino et al. 1998) which increase the probability of virus transmission. The few evidences of aggressions found in our study suggest that fighting and biting would not be a common mechanism of virus transmission among *A. azarae* males, in contrast with that observed for *Peromyscus* (Mills & Childs 1998).

The absence of differences in seroprevalence between sexes, and the social behavior of *O. flavescens* (Crespo 1966), suggest that viral transmission in this species may occurred during communal nesting in winter, as was observed for deer mice populations (Mills et al. 1999). However, Cantoni et al. (2001) observed a higher prevalence of antibodies in *O. longicaudatus* adult males than in juveniles and females, although this species also shows communal nesting. We captured few *O. flavescens*, and most of them in the winter period, so more extensive studies on this species are needed to have conclusive information about the pattern of seroprevalence variation within its populations.

The low seroprevalence observed in the *A. azarae* population in March 2001 may have been caused by a dilution of the few adults present (with high seroprevalence) within a dense population with a high proportion of juveniles (which were not infected). In March 1999, by the contrary, the population was mainly composed by adult individuals which contributed to the high seroprevalence observed.

The presence of AND Cent Lec viral lineage in the rodent populations of the area suggests that the human population is at risk of HPS disease. At present, there are no reported cases of this disease in the studied department of Exaltación de la Cruz, although there were reported cases of HPS in the neighbor departments of Zárate, Campana, Pilar and Escobar (Fig. 1). The lower human population density in Exaltación de la Cruz (25.8 inhabitants/km^2) with respect to Campana, Zárate, Pilar and Escobar (72.8, 76.2, 369.9 and 463.6, respectively; INDEC 1991) may cause a lower probability of detection of HPS cases because of a lower probability of encounter between infected rodents and humans. Nevertheless, the infection risk in Exaltación de la Cruz may be high for special demographic groups, which live or work in rural areas where rodent densities are high.

According to our results, we consider that the sanitary authorities of the area should pay special attention to the risk of cases of HPS, specially if there are changes in the land use, as was observed for Argentine hemorrhagic fever, where the etiologic agent (Junín virus) was detected in rodents years before the first human cases of the disease were reported (Parodi et al. 1961, Weissenbacher et al. 1985, Kravetz et al. 1986).

The high prevalence of Pergamino virus in *A. azarae* populations, a species widely distributed in rural and peridomestic habits of central Argentina, also represents a potential risk because we can not discard that this virus can become patogenic for humans.

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