

Human Exposure to Hantaviruses Associated with Rodents of the *Murinae* Subfamily, Madagascar

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We conducted a national human serologic study of a hantavirus detected in Madagascar rodents using a commercial kit and a new ELISA targeting the virus. Our results suggest a conservative estimate of 2.7% (46/1,680) IgG seroprevalence. A second single-district study using the new ELISA revealed a higher prevalence (7.2%; 10/139).

Hantaviruses belonging to the genus *Orthohantavirus*, family *Hantaviridae*, are frequently zoonotic. Rodents are the usual reservoirs of human pathogenic hantaviruses and typically do not show obvious signs of disease (1,2). Transmission to humans usually occurs by inhalation of aerosols contaminated with urine or feces of infected reservoir animals (3). Hantaviruses are responsible for the severe illness hemorrhagic fever with renal syndrome (HFRS) and a milder form, nephropathia epidemica (NE), as well as for hantavirus cardiopulmonary syndrome (HCPS) (1).

Recent studies have described the geographic distribution and host range of novel hantaviruses in Africa and the Indian Ocean (4–6). In Madagascar, hantavirus RNA was identified by molecular analysis

in *Rattus rattus* and *Eliurus majori* rats from a forest site in Anjozorobe district. The virus was named Anjozorobe virus (ANJZV) and is a genetic variant of Thailand orthohantavirus (THAIV) (5). In a more recent national study, Raharinosy et al. detected hantavirus RNA in 12% (n = 897) of *R. rattus* rats, and all the sequences obtained grouped with ANJZV (7), but they did not detect hantavirus RNA in *R. norvegicus* rats (0%; n = 124) (7), a species commonly associated with the cosmopolitan Seoul orthohantavirus (1). Because THAIV may cause HFRS in Southeast Asia (8), ANJZV could also be a human pathogen in Madagascar. In 1986, a limited study that used an immunofluorescence assay with Hantaan orthohantavirus (HTNV) and Puumala orthohantavirus antigens was conducted in areas around the capital and reported low titer hantavirus antibodies in the serum samples of 7/18 rat catchers in Madagascar (9).

We conducted a national study to assess hantavirus exposure in the general population of Madagascar. Sampling took place in conjunction with a recent rodent survey (7). In addition, because the original molecular hantavirus detection in Madagascar was from forest rodents (5), we also collected and analyzed human and rat samples from 4 sites close to forests.

The Study

As part of a retrospective national study on zoonoses, we collected human serum samples from 2011–2013. We then randomly recruited 1,680 asymptomatic participants (851 female and 829 male; average age 37 years; range 18–99 years). We conducted sampling in 28 sites, each with urban and rural zones;

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Table 1. Seroprevalence of hantavirus in humans in the 28 sites used for national study, Madagascar*

Site no.	Site	No. positive/total no. participants (%; 95% CI)
1	Antananarivo	8/60 (13.3; 6.3–25.1)
2	Antsirabe	0/60 (0.0; 0.0–7.5)
3	Anjozorobe	1/60 (1.7; 0.0–10.1)
4	Tsiroanomandidy	3/60 (5.0; 1.3–14.8)
5	Antsiranana	0/60 (0.0; 0.0–7.5)
6	Sambava	2/60 (3.3; 0.5–12.5)
7	Nosy-be	2/60 (3.3; 0.5–12.5)
8	Mananjary	1/60 (1.7; 0.0–10.1)
9	Ambositra	3/60 (5.0; 1.3–14.8)
10	Farafangana	4/60 (6.7; 2.1–17.0)
11	Ihosal	0/60 (0.0; 0.0–7.5)
12	Fianarantsoa	1/60 (1.7; 0.0–10.1)
13	Antsohihy	1/60 (1.7; 0.0–10.1)
14	Mandritsara	0/60 (0.0; 0.0–7.5)
15	Maevatanana	4/60 (6.7; 2.1–17.0)
16	Ambato Boeny	0/60 (0.0; 0.0–7.5)
17	Mahajanga	2/60 (3.3; 0.5–12.5)
18	Moramanga	0/60 (0.0; 0.0–7.5)
19	Toamasina	2/60 (3.3; 0.5–12.5)
20	Ambatondrazaka	0/60 (0.0; 0.0–7.5)
21	Miandrivazo	1/60 (1.7; 0.0–10.1)
22	Ejeda	2/60 (3.3; 0.5–12.5)
23	Morombe	1/60 (1.7; 0.0–10.1)
24	Toliary	2/60 (3.3; 0.5–12.5)
25	Taalagnaro	2/60 (3.3; 0.5–12.5)
26	Ambovombe	2/60 (3.3; 0.5–12.5)
27	Belo sur Tsiribihina	0/60 (0.0; 0.0–7.5)
28	Morondava	2/60 (3.3; 0.5–12.5)
Total		46/1,680 (2.7; 2.0–3.7)

*Results from IgG testing by commercial ELISA and custom ELISA developed for Anjozorobe virus.

we sampled 60 persons per site, with 30 persons per zone (10). In addition, we used samples collected during 2015–2016 from 4 rural sites close to natural forest areas in Moramanga district, which is close to Anjozorobe district. For this study, we randomly selected 139 asymptomatic participants (31–36 persons per site; average age 29 years, range 5–75 years). We also conducted trapping of the rat population in these 4 sites and randomly selected 237 *R. rattus* rats (58–61 per site).

The national ethics committee of Madagascar authorized human studies (authorization no. 066-MSANP/CE on July 26, 2011; no. 049-MSANT/CE on July 03, 2012). We conducted animal studies in accordance with Pasteur Institute animal use guidelines (<https://www.pasteur.fr/en/file/2626/download?token=YgOq4QW7>). A committee of the Institut Pasteur de Madagascar approved the studies.

For the national study, we performed initial screening using the commercial Dobrava-Hantaan IgG EIA kit (Reagent Ltd, <https://www.reagent.com>) based on the recombinant nucleocapsid (N) protein from HTNV. HTNV and THAIV, along with other *Murinae*-associated hantaviruses (Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/26/3/19-0320->

App1.pdf), exhibit close antigenic relationship (11). However, because 2-way cross-reactivity is not complete (12), we developed a new IgG ELISA based on ANJZV recombinant N protein produced by a baculovirus-mediated insect cell expression system. We used this assay to test all samples testing positive or borderline by the commercial kit and a subset of negative samples (Appendix). Based on the apparent increased detection ability of the ANJZV ELISA, we only used ANJZV ELISA for testing the human samples from the 4 sites close to forest areas.

After screening 1,680 serum samples with the commercial ELISA, we found 36 (2.1%) positive and 26 (1.5%) borderline samples. Using the custom ANJZV ELISA on these samples and a subset of 62 negative samples, we found 46 positive and 15 borderline (Appendix Tables 2, 3). Thus, the ELISA we developed specifically for ANJZV appeared to be more sensitive. To obtain a conservative estimate of seroprevalence, only samples testing positive by both assays or positive by 1 assay and borderline by the other were considered positive; testing yielded an overall prevalence of 2.7% (46/1,680; 95% CI 2.0%–3.7%) in the population; 30 male (1.8%) and 16 female (0.9%) participants tested positive.

Seropositive participants came from 20 of the 28 study sites (0–13.3% per site) distributed all over Madagascar (Table 1; Figure). Univariate generalized linear mixed models with site-zone as random effect indicated no effect of age, sex, or location (urban or rural), but we did find a slight suggestion of increased exposure in sites where our previous study (7) had detected infected rats (OR 3.0, 95% CI 0.78–11.5; $p = 0.11$).

The Moramanga sites, situated close to forest, had significantly higher seroprevalence rates (7.2%: 10/139; 95% CI 3.7%–13.2%; range 3.2%–11.1%) than the national study sites (Kruskal-Wallis test $\chi^2_1 = 4.65$; $p = 0.03$) (Table 2; Figure). This finding may partly reflect the apparent higher sensitivity of the ANJZV ELISA used for the regional study. Because 2 ($n = 62$, 3.2%) national samples tested negative by the commercial ELISA were positive by ANJZV ELISA, and 1,558 national samples were not tested by ANJZV ELISA, the overall national seroprevalence could be $>2.7\%$ ($3.2\% \times 1,558 = 50$; $(50 + 46)/1,680 = 5.7\%$). Of interest, when we tested *R. rattus* rats from the 4 Moramanga sites by nested reverse transcription PCR using a protocol described previously (Appendix) (7), we also observed significantly higher infection rates than those for the national study sites; 77 of 237 rat samples were positive (32.5%; 95% CI 26.7%–38.9%; range 19.0%–43.3%; Kruskal-Wallis test

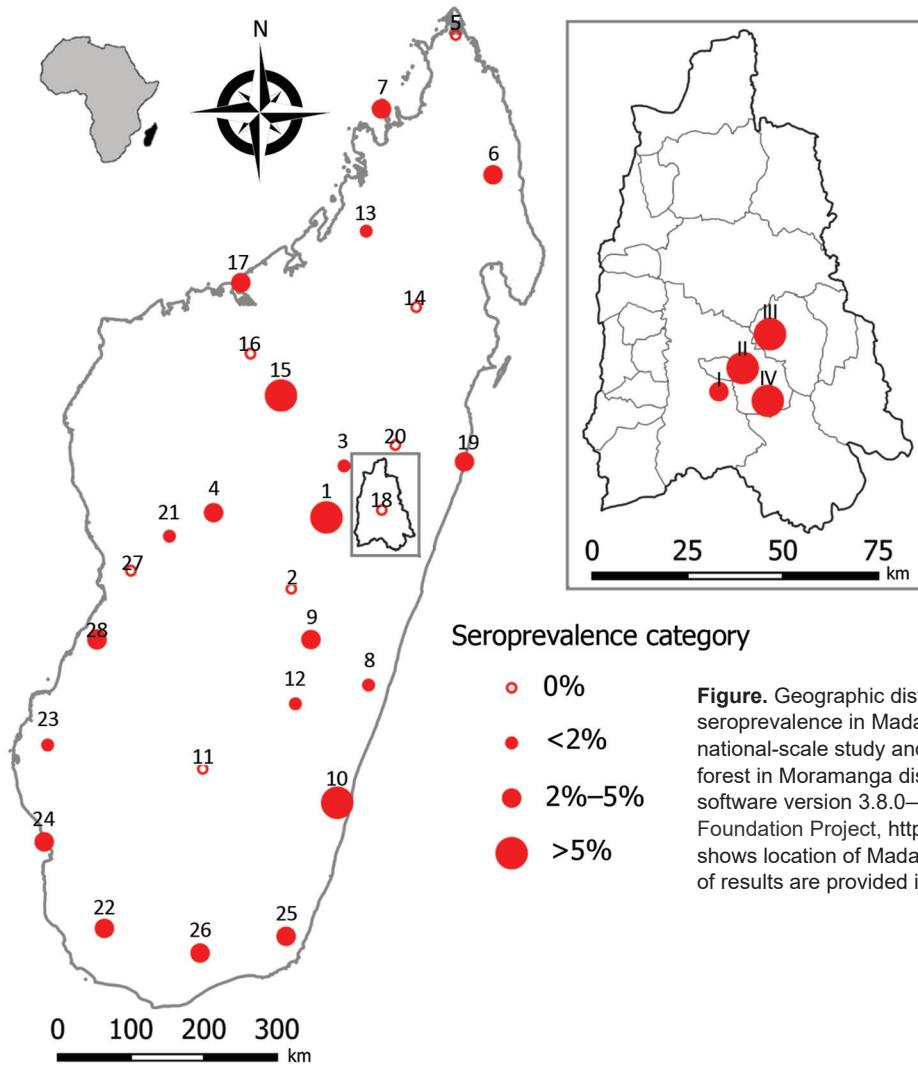


Figure. Geographic distribution of IgG hantavirus human seroprevalence in Madagascar for the 28 sites of the national-scale study and (inset) for the 4 sites close to forest in Moramanga district. Maps were built with QGIS software version 3.8.0—Zanzibar (Open Source Geospatial Foundation Project, <http://qgis.osgeo.org>). Small inset map shows location of Madagascar off the coast of Africa. Details of results are provided in Tables 1 and 2.

$\chi^2_1 = 5.55$; $p = 0.02$). These results further confirm a relatively high infection rate in the most abundant and widespread rodent in Madagascar. The small number of samples (2/61; 3%) negative by ANJZV ELISA but seropositive by the commercial ELISA could be explained by other *Murinae*-associated hantaviruses circulating in Madagascar.

Conclusions

Our results suggest the population of Madagascar is exposed to hantaviruses associated with the *Murinae* subfamily of rodents. The overall conservative prevalence estimate of 2.7% from the national-scale study, obtained using 2 ELISA assays, is similar to results from studies in some Africa countries where other confirmatory tests were used (3.9% in Cote d’Ivoire and 2.4% in the Democratic Republic of the Congo) (4,13). Although we believe some seropositive persons may have been

exposed to other *Murinae*-associated hantaviruses, considering both ELISA results in humans and rodent infection data together (7), our observations are consistent with evidence that most were exposed to ANJZV. Specifically, the ANJZV ELISA detected more seropositive persons than the commercial kit, and the cosmopolitan Seoul virus, if present in rodents in Madagascar, is at low prevalence or patchily

Table 2. Seroprevalence of hantavirus in humans in the 4 sites close to forest in Moramanga district, Madagascar

Site no.	Site	No. positive/total no. participants (%; 95% CI)
I	Mangidifoza	1/31 (3.2; 0.1–18.5)
II	Atsahatsaka	4/36 (11.1; 3.6–27.0)
III	Sahamalotra	3/36 (8.3; 2.1–23.6)
IV	Ambalafary	2/36 (5.5; 1.0–20.0)
Total		10/139 (7.2; 3.7–13.2)

*Results from IgG testing by custom ELISA developed for Anjozorobe virus.

distributed (7). Because hantavirus infection rates in *R. rattus* rats appear higher at sites close to forest, more widespread testing with the ELISA developed for Anjozorobe virus is needed to confirm whether human communities in such areas are also at higher risk for infection. In addition, hospital surveillance studies are needed in Madagascar to determine if hantavirus infection occurs in patients, with testing focused on those with fever with unknown etiology, renal failure, or both.

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Appendix

Commercial ELISA

We used Dobrava-Hantaan IgG enzyme immunoassay (Reagent Ltd, <https://www.reagent.com>) for initial screening of samples from a national-scale study in Madagascar. We chose this kit because it is a broad-spectrum assay based on recombinant nucleocapsid (N) protein from Hantaan virus (HTNV), a *Murinae*-associated hantavirus related to Anjzorobe virus (ANJZV).

Anjzorobe IgG ELISA

We developed an indirect IgG ELISA based on the use of the recombinant N protein of ANJZV. For the national study, we used this assay on all the samples testing positive or borderline by the commercial test, as well as on a subset of 62 samples tested negative by the commercial test. We selected the negative samples as matching controls (based on geographic site/zone, sex, and age) for each of the samples that were positive or borderline in the commercial assay.

Antigen Production

Sequence coding for the N protein (429 aa) of hantavirus strain Anjzorobe/Rr/MDG/2009/ATD56 (KC490916.1), flanked at N-ter by a KOZAK sequence and at C-ter by a sequence coding for an Enterokinase site (DDKC), followed by a sequence coding for a purification tag (Strep III) WSHPQFEKGGGSGGGSGGGWSHPQFEK, was synthesized and inserted into the plasmid pVL1393 (Life Technologies SAS, <https://www.thermofisher.com>). This plasmid was cotransfected with linearized baculovirus DNA bestBac2.0 (Expressions Systems, expressionsystems.com) into *Spodoptera frugiperda* (Sf9) cells. We obtained first

generations of recombinant baculoviruses P1. We obtained the recombinant protein (antigen) used for our ELISA test through infection of Sf9 cells at MOI = 5 in a 5L wave bioreactor (GE Healthcare, <https://www.gehealthcare.com>) with the recombinant baculoviruses. We harvested the supernatant after 3 days of infection and then centrifuged at 6,000 rpm for 30 min. Supernatant was concentrated on AktaFlux and treated with avidin to remove biotin in the medium and with TRIS 1M to equilibrate the pH at 8. After a centrifugation of 20,000 rpm, we filtered supernatant with a 0,2 µm filter. We purified the result based on affinity streptag with an AKTA Avant system and Steptrap-HP 1mL column (GE Healthcare).

We confirmed protein presence and quantification by SDS PAGE gel, Western blot, and Bradford assay.

Homemade Anjzorobe IgG ELISA

We saturated wells of the microtiter plate with 100 µL of recombinant Anjzorobe antigen at the concentration of 5 µg/ml diluted in carbonate buffer (coated wells); in parallel, we saturated wells with 100 µL of carbonate buffer only (uncoated wells) (Appendix Figure). In both cases, we incubated the plate for 1 hour at 37°C. We then removed the buffer, and saturated the plate with PBS blocking buffer containing 0.05% Tween20 (PBS-T 0.05%) mixed with bovine albumin serum 1% (BSA 1%) and incubated for 1 hour at room temperature. We removed the buffer and washed the plate 3 times with PBS-T 0.1%, pH 7.2.

We added 100 µL of each serum diluted to 1/400 with PBS-T buffer 0.05% – BSA 0.5% and incubated for 1 hour at 37°C. Each serum was added in duplicate wells with (coated) and without (uncoated) antigen (Appendix Figure). We then washed the plate 3 times with PBS-T 0.1%, pH 7.2; 100 µL of IgG anti-human antibody, coupled to horseradish peroxidase, diluted at 1/6000 with PBS-T buffer 0.5% – BSA 0.5%, were added and incubated for 2 hours at 37°C. After washing, we added 100 µL of ABTS peroxidase substrate and incubated for 10 min in the dark. To stop the reaction, we added sulfuric acid and measured the optical density (OD) at wavelength of 450 nm.

Analysis

Samples where duplicates in coated wells or uncoated wells had a coefficient of variation >25% for OD were repeated. For each sample, we calculated the difference between the mean OD of coated wells and the mean OD of uncoated wells (ΔOD). We defined the exposure status

of tested persons using the ratio method; this involves comparing results to negative controls, ideally incorporating controls from the study population. In the first ANJZV assay, we employed 3 negative controls (NC) (Appendix Figure), including 2 samples from healthy Malagasy participants available in our biobank and the commercial assay negative control. The threshold for each plate was mean Δ OD for NC + 3 \times standard deviation of Δ OD for NC. We calculated the mean Δ OD for NC for each individual plate, and calculated the standard deviation of Δ OD for NC over all plates run during the same week to account for variability between NC. In addition, when we detected variation between plates in the defined thresholds, we retested a subset of samples for confirmation. This included all borderline samples, as well as a subset of negative and positive samples. For these repeat assays, we included 5 negative controls obtained from healthy Malagasy participants, as well as the commercial test negative control; and a positive control corresponding to a serum sample that had consistently tested positive using the commercial test. We calculated the threshold for each plate using the ratio method as described above, but using the 5 Madagascar negative controls.

RT-PCR on *Rattus rattus* Samples

We tested RNA extracted from liver and spleen samples by nested RT-PCR using our established protocol (1), which is based on a nested RT-PCR (2). All positive samples were further confirmed using a recently developed real time RT-PCR assay based on a Taqman specific probe targeting the S sequence of hantaviruses of the Thailand group, which has been shown to have 100% specificity (3). In all cases, RNAs positive by the nested RT-PCR were positive by the Taqman real-time RT-PCR, thus indicating specificity of such results.

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Appendix Table 1. Amino acid sequence similarities of the nucleocapsid protein of hantaviruses, Madagascar

Virus	HTNV	DOBV	ANJZV	THAIV
HTNV	–	83%	85%	84%
DOBV	83%	–	83%	83%
ANJZV	85%	83%	–	97%
THAIV	84%	83%	97%	–

*ANJZV, Anjozorobe virus variant of THAIV (accession no. YP_009362283.1); DOBV, Dobrava-Belgrade orthohantavirus (accession no. AES92931.1); HTNV, Hantaan orthohantavirus (accession no. ANK77968.1); THAIV, Thailand orthohantavirus (accession no. CAL37107.1).

Appendix Table 2. Comparison of results obtained during serologic analyses using a commercial IgG hantavirus kit and a custom Anjozorobe hantavirus ELISA on the same samples*

Commercial	ANJZV			Total
	Negative	Borderline	Positive	
Negative	56	4	2	62
Borderline†	3	9	12	24
Positive	2	2	32	36
Total	61	15	46	122

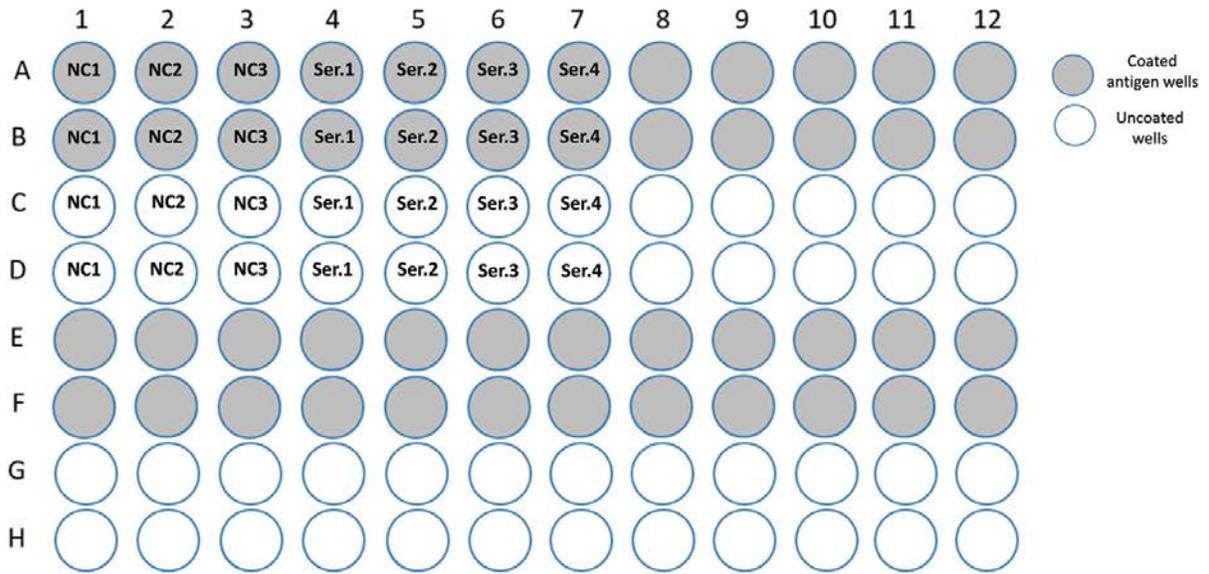
*Bold text indicates samples testing positive by both assays or borderline by one assay and positive by the other.

†Two additional samples tested borderline by commercial assay were not available for testing by the ANJZV IgG ELISA.

Appendix Table 3. Serologic results for samples detected as positive or borderline for hantavirus by commercial hantavirus ELISA kit (Reagen) and custom-developed Anjozorobe hantavirus ELISA, Madagascar*

Site no.	Site	Case				Control			
		Sex	Age, y	Reagen	ANJZV	Sex	Age, y	Reagen	ANJZV
1	Antananarivo	M	29	Positive	Positive	M	30	Negative	Negative
1	Antananarivo	M	39	Positive	Positive	M	37	Negative	Borderline
1	Antananarivo	F	23	Positive	Positive	F	23	Negative	Negative
1	Antananarivo	F	42	Positive	Positive	F	42	Negative	Negative
3	Anjozorobe	M	18	Positive	Positive	M	21	Negative	Negative
4	Tsiroanomandidy	M	22	Positive	Positive	M	21	Negative	Negative
4	Tsiroanomandidy	F	23	Positive	Borderline	F	23	Negative	Negative
6	Sambava	F	35	Positive	Negative	M	35	Negative	Negative
6	Sambava	M	45	Positive	Positive	M	46	Negative	Negative
7	Nosy-be	M	60	Positive	Positive	M	58	Negative	Negative
7	Nosy-be	F	57	Positive	Positive	F	58	Negative	Negative
8	Mananjary	M	48	Positive	Positive	M	46	Negative	Negative
9	Ambositra	F	70	Positive	Positive	M	61	Negative	Negative
9	Ambositra	M	33	Positive	Positive	M	32	Negative	Negative
9	Ambositra	M	41	Positive	Positive	M	41	Negative	Negative
10	Farafangana	M	67	Positive	Positive	M	65	Negative	Negative
12	Fianarantsoa	F	37	Positive	Positive	F	35	Negative	Negative
13	Antsohihy	M	39	Positive	Positive	M	40	Negative	Negative
15	Maevatanana	F	33	Positive	Negative	F	33	Negative	Positive
15	Maevatanana	F	23	Positive	Positive	M	23	Negative	Negative
17	Mahajanga	F	40	Positive	Positive	F	40	Negative	Negative
19	Toamasina	M	48	Positive	Positive	M	44	Negative	Negative
19	Toamasina	M	40	Positive	Positive	M	38	Negative	Negative
21	Miandrivazo	F	53	Positive	Positive	F	56	Negative	Negative
22	Ejeda	F	24	Positive	Positive	F	23	Negative	Negative
24	Toliary	M	54	Positive	Positive	M	57	Negative	Negative
24	Toliary	M	19	Positive	Positive	M	21	Negative	Negative
25	Taolagnaro	F	22	Positive	Positive	F	22	Negative	Negative
26	Ambovombe	M	44	Positive	Positive	M	45	Negative	Negative
27	Belo sur Tsiribihina	F	27	Positive	Borderline	F	24	Negative	Negative
28	Morondava	M	20	Positive	Positive	M	20	Negative	Borderline
1	Antananarivo	M	65	Borderline	Positive	M	80	Negative	Negative
1	Antananarivo	F	36	Borderline	Positive	F	35	Negative	Positive
1	Antananarivo	F	47	Borderline	Positive	F	47	Negative	Negative
4	Tsiroanomandidy	M	38	Borderline	Positive	M	42	Negative	Positive
5	Antsiranana	M	27	Borderline	Negative	M	25	Negative	Negative
6	Sambava	M	52	Borderline	Positive	M	65	Negative	Negative
9	Ambositra	M	26	Borderline	Borderline	M	26	Negative	Negative
10	Farafangana	F	38	Borderline	Positive	F	37	Negative	Negative
10	Farafangana	M	27	Borderline	Positive	M	29	Negative	Positive
10	Farafangana	M	22	Borderline	Positive	M	21	Negative	Negative
11	Ihosy	M	21	Borderline	NA	M	22	Negative	Negative
12	Fianarantsoa	M	35	Borderline	Negative	M	31	Negative	Negative
13	Antsohihy	M	34	Borderline	NA	M	36	Negative	Negative
15	Maevatanana	M	65	Borderline	Borderline	M	62	Negative	Negative
15	Maevatanana	M	61	Borderline	Positive	M	60	Negative	Negative
15	Maevatanana	F	38	Borderline	Positive	F	41	Negative	Positive
15	Maevatanana	M	25	Borderline	Positive	F	24	Negative	Negative
16	Ambato Boeny	M	100	Borderline	Negative	M	69	Negative	Negative
16	Ambato Boeny	F	18	Borderline	Borderline	F	20	Negative	Negative
17	Mahajanga	F	43	Borderline	Positive	F	43	Negative	Negative
19	Toamasina	M	56	Borderline	Negative	M	43	Negative	Negative
20	Ambatondrazaka	F	23	Borderline	Borderline	F	23	Negative	Negative
22	Ejeda	M	33	Borderline	Positive	M	31	Negative	Negative
23	Morombe	M	45	Borderline	Negative	M	44	Negative	Negative
23	Morombe	M	27	Borderline	Positive	M	27	Negative	Negative
23	Morombe	F	24	Borderline	Borderline	F	23	Negative	Negative
24	Toliary	F	30	Borderline	Borderline	F	31	Negative	Negative
25	Taolagnaro	M	26	Borderline	Positive	M	30	Negative	Negative
26	Ambovombe	M	76	Borderline	Positive	M	62	Negative	Negative
27	Belo sur Tsiribihina	M	28	Borderline	Borderline	M	29	Negative	Negative
28	Morondava	M	18	Borderline	Positive	M	18	Negative	Negative

*Samples testing negative by commercial ELISA matched by site/zone, sex, and age. ANJZV, Anjozorobe orthohantavirus ELISA; NA, not available.



Appendix Figure. Schematic plan of plate used in Anjzorobe hantavirus ELISA. Gray circles indicate wells containing recombinant Anjzorobe antigen. White circles indicate wells with buffer solution only. Ser., serum.