Short Report: Seroprevalence of Human Leptospirosis in Reunion Island (Indian Ocean) Assessed by Microscopic Agglutination Test on Paper Disc-Absorbed Whole Blood

Amélie Desvars, Jimmy Gigan, Géraldine Hoarau, Patrick Gérardin, François Favier, and Alain Michault*

Unité Mixte de Recherche Contrôle des Maladies Animales Exotiques et Emergentes (UMRCMAEE), Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Sainte Clotilde, La Réunion, France;
Laboratoire de Bactériologie—Parasitologie—Virologie—Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint Pierre, La Réunion, France; Centre d'Investigation Clinique—Epidémiologie Clinique (CIC-EC) de La Réunion, Institut National de la Santé et de la Recherche Médicale (INSERM)/CHR/Université de La Réunion/Union Régionale des Médecins Libéraux de La Réunion (URMLR), GHSR, CHR, Saint Pierre, La Réunion, France

Abstract. In the last decade, leptospirosis has emerged as a globally important infectious disease. Humans most commonly become infected through occupational, recreational, or domestic contact with the urine of carrier animals, either directly or through contaminated water or soil. The disease occurs in urban areas of industrialized and developing countries as well as rural regions worldwide. We present a retrospective study conducted in 2006 on 2,269 randomly selected Reunion Island inhabitants. Blood sampling was performed on individual blotting papers, and microscopic agglutination test (MAT) was conducted on paper disc-absorbed (PDA) blood. We showed that seroprevalence of leptospirosis was $0.66\% \pm 0.34$ in the global population of Reunion Island, which is 1.78 lower than the seroprevalence estimated 20 years before. The serological method is described, and the results discussion focuses on methodology and socio-economic factors.

Leptospirosis is a potentially life-threatening zoonosis with a wide geographic distribution occurring in many mammalian species, including humans. The disease is caused by pathogenic serovars of *Leptospira* spp., a spirochete bacteria. The pathogenesis is systemic and associated with fever and renal, hepatic, pulmonary, and reproductive dysfunctions. The clinical spectrum is broad and not specific, whereas the majority of cases are probably unapparent.¹ Diagnosis is routinely based on the microscopic agglutination test (MAT), which is actually recognized to be serogroup-specific. Reunion Island is a French department located in the Indian Ocean, where the incidence of leptospirosis is high, with 8.69 cases per 100,000 inhabitant per year in 2009.² Nevertheless, because of the great number of paucisymptomatic forms, reported cases underestimate the real incidence of leptospirosis. In 1987, a large serosurvey based on a large sample of 3,338 Reunion Islanders estimated a community seroprevalence of 1.18% at the seropositivity cutoff of 1/100.³ In the present study, using the blotting papers collected on the sample of the population-based SEROCHIK survey (a previous serosurvey aimed at assessing the post-epidemic attack rate of Chikungunya fever in 2006),⁴ we report a simple and original method to estimate the magnitude of seroprevalence of human leptospirosis in 2006 in a large random sample of 2,269 individuals. We compare our results for 2006 with the seroprevalence rate found in 1987^{3,5} and discuss risk factors that can influence the prevalence of leptospirosis in Reunion Island.

The 2,442 participants of the SEROCHIK survey belonged to a random sample consisting of a two-stage sampling. At the first-stage sampling, households were randomly selected by the Institut National de la Statistique et des Etudes Economiques (INSEE) over 23 townships, covering the entire territory of the island (except the Mafate circus). At the second-stage sampling, one participant per household was selected accord-

ing to the Kish method.⁴ For each individual, blood was taken with a finger stick on individual blotting papers (Whatman 903 ProteinSaver Card, Whatman, Dassel, Germany). The paper was air-dried before storing at -20°C until analysis.

To optimize the serological method using MAT on paper disc-absorbed (PDA) blood, we tested different technical parameters on 22 seropositive and 30 seronegative patients, who were previously diagnosed (from 2002 to 2005) by classical MAT, conducted on fluid serum at our laboratory. Among the seropositive patients, 11 were seropositive at titer 1/200 and 11 were seropositive at titer 1/6,400 with classical MAT. We used the classical MAT, which is the serological method of reference, to evaluate the MAT on PDA blood, and we compared antibody titers obtained for the 52 previously diagnosed patients with both methods. Whole blood from each positive patient was first absorbed within the specific circular area on a blotting paper. Then, the remaining blood was centrifuged, and the serum used for a second analysis with classical MAT. A circular area was cut in the PDA blood, and the blood was eluted by soaking overnight at 4°C in a 2-mL microtube with Sorensen buffer at the dilution 1:25 or with a 50/50 mix of Sorensen buffer and phosphate buffer saline (PBS). Two circular surfaces ($S = r^2\pi$) of the PDA blood were tested: $S_1 = 1.5^2\pi$ and $S_2 = 2.5^2\pi$ mm². The volume of elution buffer was adjusted depending on the surface of the PDA blood considered: 288 μL for S_1 and 800 μL for S_2 . Then, elution of the PDA blood samples was completed by either leaving the PDA blood in elution buffer one night at 4°C or leaving it 2 hours at room temperature with slow shaking or grinding for 2 minutes at 30 Hz in a vibratory shaker (Mixer mill MM 301, Retsch GmbH, Haan, Germany). Then, samples were centrifuged for 15 minutes at 10,000 rpm (25°C). The supernatant was considered equivalent to a 1:100 dilution of serum and tested in the same way as fresh serum by classical MAT.^{6,7}

To estimate the seroprevalence of leptospirosis in the global population, the MAT was conducted on 2,269 PDA blood samples collected in the population described above. The antigens used were live cultures of *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain isolated in Reunion Island, *L. interrogans* serogroup Pyrogenes serovar

*Address correspondence to Alain Michault, Laboratoire de Bactériologie—Parasitologie—Virologie—Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), BP350, Avenue Mitterrand Saint Pierre cedex 97448, La Réunion, France. E-mail: alain.michault@chr-reunion.fr

TABLE 1
Organisms used as capture antigens in the MAT represent four different serogroups

Genus	Species	Serogroup	Serovar	Strain
<i>Leptospira</i>	<i>interrogans</i>	Icterohaemorrhagiae	Copenhageni	Strain isolated in Reunion Island
<i>Leptospira</i>	<i>interrogans</i>	Pyrogenes	Pyrogenes	Salinem
<i>Leptospira</i>	<i>borgpetersenii</i>	Sejroe	Sejroe	M84
<i>Leptospira</i>	<i>biflexa</i>	Semarang	Patoc	Patoc I

Pyrogenes strain Salinem, and *L. borgpetersenii* serogroup Sejroe serovar Sejroe strain M84 (Table 1). We choose strains representative of three serogroups (Icterohaemorrhagiae, Pyrogenes, and Sejroe), because they are the most frequently diagnosed serogroups in clinical cases in Reunion Island; also, one locally isolated strain was chosen, because antibody titers to local isolates are often higher than titers to laboratory stock strains of serovars within the same serogroup.¹ The non-pathogenic strain *L. biflexa* serogroup Semarang serovar Patoc strain Patoc I (Table 1) was also included in the panel, because it cross-reacts with many pathogenic serogroups,⁸ allowing the detection of antibodies against not tested serogroups.

We found that using Sorensen buffer 1:25 without PBS was better on the antibody titers for both serological methods. Leaving the PDA blood sample one night at 4°C was not efficient to elute the antibodies, whereas grinding the sample or leaving it 2 hours at room temperature with slow shaking was equivalent. Thus, we chose the grinding method, because it was saved. We observed that, for the same positive patient, results with the circular surfaces S1 and S2 were similar. Thus, we chose to use the circular surface S1 ($r = 1.5$ mm), because it used a smaller surface of PDA blood.

All negative patients tested with the classical MAT method on fresh serum were negative with the method on PDA blood. Antibody titers against leptospirosis obtained with the MAT conducted on PDA blood for the 11 patients positive at titer 1/6,400 were one- to two-fold dilutions lower in titers compared with positivity obtained with MAT on fresh serum. In contrast, antibody titers for the 11 patients positive at titer 1/200 were equal or one-fold dilution lower (Table 2).

Because cross-reactions between serogroups are common as well as paradoxical reactions, in which the initial immune response is directed to a heterologous serovar or serogroup, MAT is of little value for the identification of the infecting serovar in individual cases of leptospirosis.⁹ In consequence,

our study presents only the seroprevalence of the disease and not the potential infecting serovars.

The seroprevalence of leptospirosis in the global population of Reunion Islanders in 2006, evaluated on a sample of 2,269 persons, was $0.66\% \pm 0.34$ (95% confidence interval) at the seropositivity cutoff of 1/100. This seroprevalence was 1.78 times lower than that reported by Duval and others³ in 1987. Moreover, the incidence of the disease in 2006 was 7.52 cases per 100,000 inhabitants per year² (i.e., 2.05 times lower than in 1987).⁵

During this 20-year period and still now, Icterohaemorrhagiae remains the serogroup involved in the majority of the clinical cases in Reunion Island.^{2,3} We limited the number of tested serogroups to four; thus, if non-included serogroups with titers close to the threshold of positivity are circulating in the population of Reunion Island, they were not detected by our MAT. Nevertheless, major serogroups found in symptomatic patients were similar in 1987 and 2006 (Icterohaemorrhagiae, Pyrogenes, and Sejroe)^{3,10}; in consequence, if minor serogroups were circulating in 2006, they would not affect the global seroprevalence of leptospirosis in the population.

Several socioeconomic factors may explain the decrease in the seroprevalence of leptospirosis during 19 years in Reunion Island. The first factor is the distribution of the population with regard to climatic factors. Indeed, rainfall is very abundant in the east ($> 2,000$ mm/year), whereas the north and south count less than 2,000 mm/year; also, the western coastal region counts less than 100 mm/year. Rainfall favors indirect contamination.¹ Between 1987 and 2006, the population of the west side of Reunion Island increased from 1.4% to 4%,¹¹ whereas there was a global decrease in the population of the east side. In consequence, the relative percentage of the population exposed to the climatic risk factor decreased between 1987 and 2006.

Second, changes in the occupational activities of the population may have influenced the seroprevalence of the disease. Working in fields or outdoors is a well-known risk factor of contamination for leptospirosis,^{3,12} whereas office workers are less exposed. In 1987, the service sector used less than 75% of working people against 85% in 2006.¹¹ Moreover, in 2006, Reunion Island counted only 7.7% of farm workers versus 14% in 1987.¹¹ In consequence, in two decades, the number of people daily exposed to the environmental reservoir of the disease decreased greatly.

Third, housing in Reunion Island has changed dramatically between 1987 and 2006, with an increase in the number of families living in apartments: 17.9% in 1987 versus 28.4% in 2006.¹¹ Gardening and presence of rodents in the living environment are risk factors for acquiring clinical leptospirosis,¹³ whereas living in an apartment is a protecting factor of contamination.¹² Moreover, the improvement of sanitary living conditions in Reunion Island (23.9% of the households with no running water, toilets, or bathroom in 1987 versus 5% in

TABLE 2

Results of the MAT conducted on PDA blood on the 22 positive patients

Serum	Positive patients at dilution 1/6,400*	Positive patients at dilution 1/200*
1	Positive 1/3,200	Positive 1/200
2	Positive 1/1,600	Positive 1/200
3	Positive 1/1,600	Positive 1/200
4	Positive 1/1,600	Positive 1/200
5	Positive 1/3,200	Positive 1/200
6	Positive 1/1,600	Positive 1/200
7	Positive 1/3,200	Positive 1/200
8	Positive 1/1,600	Positive 1/200
9	Positive 1/1,600	Positive 1/200
10	Positive 1/1,600	Positive 1/100
11	Positive 1/1,600	Positive 1/100

*With classical MAT conducted on fresh serum.

2006)¹¹ as well as recurrent awareness campaigns about the disease among the population may have also participated in the reduction of the incidence of the disease.

In conclusion, we have shown that the PDA whole-blood sample method offers convenience in the collection, holding, and shipping of samples. This method can be very useful for large-scale studies or even for studies in the children population, because the sampling is not invasive and easy to perform. The method can be easily used in developing countries because of its cheapness. Also, conservation of the blood before analysis does not require immediate refrigeration. We have also evidenced that prevalence of leptospirosis has declined in Reunion Island between 1987 and 2006 and that it could be explained by socio-economic factors linked to sanitary improvements and the transition from a rural to urban way of life.

Received July 20, 2011. Accepted for publication August 25, 2011.

Acknowledgments: The authors thank Morgane Chambost and Mathieu Nicole for their contribution to this work and Vincent Michault for his help on the manuscript.

Authors' addresses: Amélie Desvars, Unité Mixte de Recherche Contrôle des Maladies Animales Exotiques et Emergentes (UMRCMAEE), Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Sainte Clotilde, La Réunion, France and Laboratoire de Bactériologie–Parasitologie–Virologie–Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint Pierre, La Réunion, France, E-mail: amel.desvars@gmail.com. Jimmy Gigan, Géraldine Hoarau, and Alain Michault, Laboratoire de Bactériologie–Parasitologie–Virologie–Hygiène, Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint Pierre, La Réunion, France, E-mails: jimmy.gigan@chr-reunion.fr, guynoelhoa rau@gmail.com and alain.michault@chr-reunion.fr. Patrick Gérardin and François Favier, Centre d'Investigation Clinique–Epidémiologie Clinique (CIC–EC) de La Réunion, Institut National de la Santé et de la Recherche Médicale (INSERM)/CHR/Université de La Réunion/Union Régionale des Médecins Libéraux de La Réunion (URMLR), Groupe Hospitalier Sud Réunion (GHSR), Centre Hospitalier Régional (CHR), Saint Pierre, La Réunion, France, E-mails: patrick.gerardin@chr-reunion.fr and francois.favier@chr-reunion.fr.

REFERENCES

1. Levett PN, 2001. Leptospirosis. *Clin Microbiol Rev* 14: 296–326.
2. Institut Pasteur, 2010. *Rapports d'activité du Centre National de Référence des Leptospiroses*. Available at: <http://www.pasteur.fr/ip/easysite/go/03b-000031-010/actualites-rapports>. Accessed December 21, 2010.
3. Duval G, Michault A, Baranton G, Law-Koune JD, Folio G, Bertil G, Guiserix J, 1991. Sero epidemiological survey on human leptospirosis in Reunion Island. *Rev Epidemiol Sante Publique* 39: 135–141.
4. Gérardin P, Guernier V, Perrau J, Fianu A, Le Roux K, Grivard P, Michault A, de Lamballerie X, Flahault A, Favier F, 2008. Estimating Chikungunya prevalence in La Réunion Island outbreak by serosurveys: two methods for two critical times of the epidemic. *BMC Infect Dis* 8: 99.
5. Law-Koune JD, Duval G, Michault A, Baranton G, De Camaret P, Lemahieu JM, Van-der-Linden T, 1989. Human leptospirosis in Reunion Island: epidemiological study during three years (1985–1987). *Bull Soc Pathol Exot* 82: 185–191.
6. Fianu A, Le Roux K, Grivard P, Le Pors AK, Gigan J, Michault A, Gérardin P, Favier F, 2007. Valeur discriminante du test de dépistage du Chikungunya sur buvard. *Bull Soc Pathol Exot* 100: 356–357.
7. Kingscote BF, 1969. Leptospirosis survey in a white-tailed deer herd in Ontario: comparative use of fluid and paper disc-absorbed blood. *J Wildl Dis* 5: 68–72.
8. Faine S, Adler B, Bolin C, Pérolat P, 1999. *Leptospira and Leptospirosis*. Melbourne, Australia: MediSci.
9. Levett PN, 2003. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clin Infect Dis* 36: 447–452.
10. Centre National de Référence des Leptospiroses, 2007. *Activity Report 2006*. Paris, France: Institut Pasteur.
11. Institut National de la Statistique et des Etudes Economiques, 2010. *La Revue Économie de La Réunion*. Available at: <http://www.insee.fr/fr>. Accessed January 26, 2011.
12. Sarkar U, Nascimento S, Barbosa R, Martins R, Nuevo H, Kalafanos I, Grunstein I, Flannery B, Dias J, Riley LW, Reis MG, Ko AI, 2002. Population-based case-control investigation of risk factors for leptospirosis during an urban epidemic. *Am J Trop Med Hyg* 66: 605–610.
13. Bovet P, Yersin C, Mérien F, Davis CE, Pérolat P, 1999. Factors associated with clinical leptospirosis: a population-based case-control study in the Seychelles (Indian Ocean). *Int J Epidemiol* 28: 583–590.