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Evaluation of Four Whole-Cell *Leptospira*-Based Serological Tests for the Diagnosis of Urban Leptospirosis

Running title: Rapid Serological Testing for Urban Leptospirosis

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23 **ABSTRACT**

24 Four serologic assays for leptospirosis had a sensitivity of 72-88% and specificity of 88-
25 100% in the setting of high endemic urban transmission, indicating that these assays in ELISA
26 and rapid formats may be used as an alternative to the microscopic agglutination test for urban
27 leptospirosis. Testing of a second sample will be required in cases with an initial negative result
28 since sensitivity was low (46-68%) during the first week of illness.

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31 **KEYWORDS**: leptospirosis; diagnosis; *Leptospira*, serology, urban epidemics; commercial
32 assays

NOTE

Leptospirosis is a major public health problem in developing countries where endemic transmission and outbreaks of this spirochetal disease causes high mortality and morbidity (4, 18, 21). Leptospirosis produces a spectrum of clinical manifestations which range from a mild febrile illness to severe disease forms such as Weil's syndrome and severe pulmonary haemorrhage syndrome (9, 10). Case fatality for severe leptospirosis is 5-40%, respectively (4, 9, 10, 21). Prompt identification of leptospirosis is needed, as antibiotic therapy provides greatest benefit when administered early in the infection (9, 38).

Timely diagnosis will need to rely on an effective laboratory test since the presentation of early-phase leptospirosis is often non-specific (4, 18, 21). Misdiagnosis is a major problem in regions where other causes of undifferentiated febrile illness and hemorrhagic fever are endemic (12, 14, 17, 23, 37, 39). The microscopic agglutination test (MAT), the standard for diagnostic confirmation, is impractical for clinical decision making since it is requires analysis of paired serum samples for proper interpretation and a reference laboratory to perform dark field microscopy (9, 18). Whole-*Leptospira*-based serologic assays in ELISA and rapid formats have been developed as a more feasibly employed alternative. Large evaluations, several of which were multi-center trials, found that the sensitivity and specificity of these tests ranged from 28-72% and 10-99%, respectively (2, 8, 13, 19, 31, 33-35). Performance varies significantly across geographical regions, indicating that these assays need to be validated for distinct epidemiological situations. Furthermore, recent studies have found that their performance was poor in settings of high endemic rural transmission of leptospirosis (5, 24, 29, 36).

Leptospirosis has emerged to become an urban health problem as slum settlements have grown worldwide and outbreaks in these communities are increasingly reported (4, 15, 16, 21, 28,

30). To date a side-by-side comparison of commercially available whole-*Leptospira*-based serologic tests has not been performed in defined populations from areas of high endemic urban transmission. Herein we report the performance of four serologic assays in patient and control subject groups from the city of Salvador, Brazil where incidence of severe leptospirosis is 10 per 100,000 population (16, 30).

A serum panel was created from leptospirosis cases identified during active surveillance and healthy blood bank donors. Acute-phase samples were evaluated for 96 cases which were randomly selected among the 296 cases who were identified between 2000 and 2005, provided paired serum samples and had MAT and culture isolation criteria for confirmed leptospirosis (16). We excluded cases that were confirmed on the basis of a single acute-phase sample since these patients presented later in the course of their illness and their inclusion would introduce sample bias. The serum panel included 29 acute-phase samples collected less than seven days from the onset of illness. To obtain a statistically solid number of sera to specifically explore the performance of the tests in this clinically important early stage of the disease, an additional group of 43 randomly-selected cases was included. Convalescent-phase samples were evaluated from 50 randomly-selected leptospirosis cases of the 96 cases. Sera from 80 blood bank donors who were residents of Salvador during the surveillance period were evaluated as control samples. The evaluation of the serum panel (N=269) was conducted in a double-blinded manner. The *Leptospira* IgM ELISA and Dip-S-tick (PanBio Inc.), LeptoTek Dri-Dot (Biomérieux), and EIE-IgM-Leptospirose (Bio-Manguinhos, Oswaldo Cruz Foundation) assays were performed according to the manufacturers' instructions. All assays detected IgM antibodies except the Dri-Dot test, which detected *Leptospira*-specific antibodies. Sensitivity and specificity was defined respectively, as the proportion of samples from leptospirosis patients which were positive and the

79 proportion of samples from healthy individuals which were negative according to the criteria
80 stipulated for each kit. EpiInfo version 3.3 (Centers for Disease Control and Prevention) was
81 used to calculate 95% confidence intervals for sensitivity and specificity estimates and determine
82 significant differences (P<0.05) according to the chi-square test. The study protocol was
83 approved by the IRB committees of the Oswaldo Cruz Foundation-Brazilian Ministry of Health
84 and the Weill Medical College of Cornell University.

85 The sensitivity of the assays in identifying acute-phase leptospirosis ranged from 72 to
86 88% (Table). The mean interval between onset of illness and time of collection of acute-phase
87 samples was 8.5 ± 3.8 days. There were no significant differences between the sensitivities of the
88 four assays. The assays had greater sensitivity than that for the MAT criteria of a titer ≥1:100
89 which is recommended as a screening criteria during acute-phase illness (38). The sensitivity of
90 the assays increased to 80-96% for convalescent-phase samples which were collected 23.3 ± 7.4
91 days after the onset of illness (Table). Specificity of the assays was in general high, although the
92 PanBio ELISA had lower specificity (88%) in comparison to the other assays (95-100%) (Table).

93 Overall concordance between the four serologic assays was good (kappa >0.67) in testing
94 acute and convalescent-phase samples from leptospirosis cases and healthy subject samples.
95 Concordance between the *Leptospira* IgM ELISA, LeptoTek Dri-Dot and EIE-IgM-Leptospirose
96 with the MAT screening criteria was moderate to good (kappa = 0.41-0.90) for all three sample
97 groups. However, agreement between the Dip-S-tick and the MAT screening was poor (kappa =
98 0.34) when testing sera collected less than seven days after onset of illness.

99 The sensitivities of the four assays were 33-67% in diagnosing leptospirosis with acute-
100 phase samples that were obtained less than seven days from the onset of symptoms (Table).
101 When results were stratified further, the assays had a sensitivity of 33-52% and 45-78% in

detecting leptospirosis in the 2nd to 4th and 5th to 7th days of illness, respectively (Fig.). The PanBio ELISA had the highest sensitivity during these intervals.

The study findings indicate that available whole-*Leptospira*-based serological assays are useful methods for the laboratory diagnosis of urban leptospirosis. We found similar overall performance characteristics in the urban endemic setting as that previously reported in large multi-center evaluations (33, 34). Despite limitations with respect to sensitivity during early-phase illness, the assays have high specificity and will be more feasibly implemented than the MAT. Our findings differ with those of recent evaluations which showed that whole-*Leptospira* assays had low specificity in rural regions with high endemic transmission (5, 7, 35, 36). These discrepancies most likely relate to differences in the timing of the collection of serum samples and/or proportions of patients who experience primary and secondary *Leptospira* infections among the studies. Alternatively, the differences may relate to the etiologic serovar(s) associated with rural and urban settings. Copenhageni is the most frequently reported serovar for urban leptospirosis (16, 25, 27, 28) whereas rural leptospirosis is often due to concurrent transmission of several serovars (9, 18). Our evaluation was conducted from a single site and therefore may not necessarily apply to other endemic urban settings. However, the study's findings are consistent with those of evaluations of in-house assays or individual commercial kits which were performed with predominantly urban-based subjects from other sites (3, 22, 25, 28, 32).

Although the assays used different formats and antigen preparations, they demonstrated similar sensitivity and specificity. This finding is not unexpected, since whole-*Leptospira*-based assays appear to detect antibodies against immunodominant carbohydrate epitopes, such as broad reactive antigen (1, 9, 20). The type of assay format, whether ELISA or rapid detection-based, was not associated with significant increase in performance. Selection of an assay will therefore

rely more on availability, cost and the feasibility of implementing the test for point-of-care diagnosis. It is important to note that screening assays do not discriminate infections due to different infecting serogroups. Additional diagnostic methods, such as culture isolation and the MAT, will continue to be required in order to monitor changes in circulating serogroups that may occur during surveillance.

The major limitation of whole-*Leptospira*-based serological assays is the low sensitivity (<67%) in the first week of illness. This finding appears to be a widely-observed phenomenon across geographical regions (2, 7, 13). In our study, the assays' sensitivity increased to >90% in leptospirosis patients who presented with 8-10 days of illness (Fig.). Therefore testing of a second sample, such as is done in dengue, is recommended for suspected cases with an initial negative or doubtful result. Nevertheless, there is a continued need to develop new diagnostic approaches, such as recombinant protein-based serological tests and antigen capture assays, which detect leptospirosis early in the course of illness and can be feasibly applied in point-of-care settings (6, 11, 26). Such assays may form the basis of public health responses that aim to initiate timely therapeutic interventions and reduce high mortality due to severe disease forms.

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REFERENCES

1. **Adler, B., and S. Faine.** 1978. The antibodies involved in the human immune response to leptospiral infection. J. Med. Microbiol. **11:**387-400.

2. **Bajani, M. D., D. A. Ashford, S. L. Bragg, C. W. Woods, T. Aye, R. A. Spiegel, B. D. Plikaytis, B. A. Perkins, M. Phelan, P. N. Levett, and R. S. Weyant.** 2003. Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. J. Clin. Microbiol. **41:**803-809.

3. **Bharadwaj, R., A. M. Bal, S. A. Joshi, A. Kagal, S. S. Pol, G. Garad, V. Arjunwadkar, and R. Katti.** 2002. An urban outbreak of leptospirosis in mumbai, India. Jpn. J. Infect. Dis. **55:**194-196.

4. **Bharti, A. R., J. E. Nally, J. N. Ricaldi, M. A. Matthias, M. M. Diaz, M. A. Lovett, P. N. Levett, R. H. Gilman, M. R. Willig, E. Gotuzzo, and J. M. Vinetz.** 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis. **3:**757-771.

5. **Blacksell, S. D., L. Smythe, R. Phetsouvanh, M. Dohnt, R. Hartskeerl, M. Symonds, A. Slack, M. Vongsouvath, V. Davong, O. Lattana, S. Phongmany, V. Keolouangkot, N. J. White, N. P. Day, and P. N. Newton.** 2006. Limited diagnostic capacities of two commercial assays for the detection of *Leptospira* immunoglobulin M antibodies in Laos. Clin. Vaccine Immunol. **13:**1166-1169.

6. **Croda, J., J. G. Ramos, J. Matsunaga, A. Queiroz, A. Homma, L. W. Riley, D. A. Haake, M. G. Reis, and A. I. Ko.** 2007. *Leptospira* Immunoglobulin-Like (Lig) proteins is a Serodiagnostic Marker for Acute Leptospirosis. J. Clin. Microbiol. **45:**1528-1534.

7. **Effler, P. V., A. K. Bogard, H. Y. Domen, A. R. Katz, H. Y. Higa, and D. M. Sasaki.** 2002. Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. J. Clin. Microbiol. **40:**1464-1469.

8. **Effler, P. V., H. Y. Domen, S. L. Bragg, T. Aye, and D. M. Sasaki.** 2000. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis in Hawaii. J. Clin. Microbiol. **38:**1081-1084.

9. **Faine, S. B., B. Adler, C. Bolin, and P. Perolat.** 1999. *Leptospira* and leptospirosis, 2nd ed. MediSci, Melbourne, Australia.

10. **Farr, R. W.** 1995. Leptospirosis. Clin. Infect. Dis. **21:**1-8.

11. **Flannery, B., D. Costa, F. P. Carvalho, H. Guerreiro, J. Matsunaga, E. D. da Silva, A. G. Ferreira, L. W. Riley, M. G. Reis, D. A. Haake, and A. I. Ko.** 2001. Evaluation of recombinant Leptospira antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J. Clin. Microbiol. **39:**3303-3310.

12. **Flannery, B., M. M. Pereira, L. d. F. Velloso, C. d. C. Carvalho, L. G. de Codes, G. d. S. Orrico, C. M. Dourado, L. W. Riley, M. G. Reis, and A. I. Ko.** 2001. Referral pattern of leptospirosis cases during a large urban epidemic of dengue. Am. J. Trop. Med. Hyg. **65:**657-663.

13. **Hull-Jackson, C., M. B. Glass, M. D. Ari, S. L. Bragg, S. L. Branch, C. U. Whittington, C. N. Edwards, and P. N. Levett.** 2006. Evaluation of a commercial latex agglutination assay for serological diagnosis of leptospirosis. J. Clin. Microbiol. **44:**1853-1855.

14. **Ismail, T. F., M. O. Wasfy, B. Abdul-Rahman, C. K. Murray, D. R. Hospenthal, M. Abdel-Fadeel, M. Abdel-Maksoud, A. Samir, M. E. Hatem, J. Klena, G. Pimentel, N.**

El-Sayed, and R. Hajjeh. 2006. Retrospective serosurvey of leptospirosis among patients with acute febrile illness and hepatitis in Egypt. Am. J. Trop. Med. Hyg. **75:**1085-1089.

15. **Karande, S., H. Kulkarni, M. Kulkarni, A. De, and A. Varaiya.** 2002. Leptospirosis in children in Mumbai slums. Indian J. Pediatr. **69:**855-858.

16. **Ko, A. I., M. Galvao Reis, C. M. Ribeiro Dourado, W. D. Johnson, Jr., and L. W. Riley.** 1999. Urban epidemic of severe leptospirosis in Brazil. Lancet **354:**820-825.

17. **LaRocque, R. C., R. F. Breiman, M. D. Ari, R. E. Morey, F. A. Janan, J. M. Hayes, M. A. Hossain, W. A. Brooks, and P. N. Levett.** 2005. Leptospirosis during dengue outbreak, Bangladesh. Emerg. Infect. Dis. **11:**766-769.

18. **Levett, P. N.** 2001. Leptospirosis. Clin. Microbiol. Rev. **14:**296-326.

19. **Levett, P. N., S. L. Branch, C. U. Whittington, C. N. Edwards, and H. Paxton.** 2001. Two methods for rapid serological diagnosis of acute leptospirosis. Clin. Diagn. Lab. Immunol. **8:**349-351.

20. **Matsuo, K., E. Isogai, and Y. Araki.** 2000. Utilization of exocellular mannan from *Rhodotorula glutinis* as an immunoreactive antigen in diagnosis of leptospirosis. J. Clin. Microbiol. **38:**3750-3754.

21. **McBride, A. J., D. A. Athanazio, M. G. Reis, and A. I. Ko.** 2005. Leptospirosis. Curr. Opin. Infect. Dis. **18:**376-386.

22. **McBride, A. J. A., F. A. Pereira, E. D. da Silva, R. B. de Matos, E. D. da Silva, A. G. P. Ferreira, M. G. Reis, and A. I. Ko.** 2007. Evaluation of the EIE-IgM Leptospirose assay for the serodiagnosis of leptospirosis. Acta Trop. **102:**206-211.

23. **Murdoch, D. R., C. W. Woods, M. D. Zimmerman, P. M. Dull, R. H. Belbase, A. J. Keenan, R. M. Scott, B. Basnyat, L. K. Archibald, and L. B. Reller.** 2004. The

etiology of febrile illness in adults presenting to Patan hospital in Kathmandu, Nepal. Am. J. Trop. Med. Hyg. **70:**670-675.

24. **Myint, K. S., R. V. Gibbons, C. K. Murray, K. Rungsimanphaiboon, W. Supornpun, R. Sithiprasasna, M. R. Gray, C. Pimgate, M. P. Mammen, Jr., and D. R. Hospenthal.** 2007. Leptospirosis in Kamphaeng Phet, Thailand. Am. J. Trop. Med. Hyg. **76:**135-138.

25. **Ooteman, M. C., A. R. Vago, and M. C. Koury.** 2006. Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. J. Microbiol. Methods **65:**247-257.

26. **Palaniappan, R. U., Y. F. Chang, F. Hassan, S. P. McDonough, M. Pough, S. C. Barr, K. W. Simpson, H. O. Mohammed, S. Shin, P. McDonough, R. L. Zuerner, J. Qu, and B. Roe.** 2004. Expression of leptospiral immunoglobulin-like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA. J. Med. Microbiol. **53:**975-984.

27. **Pereira, M. M., M. G. S. Matsuo, A. R. Bauab, S. A. Vasconcelos, Z. M. Moraes, G. Baranton, and G. I. Saint.** 2000. A clonal subpopulation of *Leptospira interrogans* sensu stricto is the major cause of leptospirosis outbreaks in Brazil. J. Clin. Microbiol. **38:**450-452.

28. **Romero, E. C., C. C. Bernardo, and P. H. Yasuda.** 2003. Human leptospirosis: a twenty-nine-year serological study in Sao Paulo, Brazil. Rev. Inst. Med. Trop. Sao Paulo **45:**245-248.

29. **Russell, K. L., G. M. A. Montiel, D. M. Watts, R. C. Lagos-Figueroa, G. Chauca, M. Ore, J. E. Gonzalez, C. Moron, R. B. Tesh, and J. M. Vinetz.** 2003. An outbreak of leptospirosis among Peruvian military recruits. Am. J. Trop. Med. Hyg. **69:**53-57.

30. **Sarkar, U., S. F. Nascimento, R. Barbosa, R. Martins, H. Nuevo, I. Kalafanos, I. Grunstein, B. Flannery, J. Dias, L. W. Riley, M. G. Reis, and A. I. Ko.** 2002. Population-based case-control investigation of risk factors for leptospirosis during an urban epidemic. Am. J. Trop. Med. Hyg. **66:**605-610.

31. **Sehgal, S. C., P. Vijayachari, A. P. Sugunan, and T. Umapathi.** 2003. Field application of Lepto lateral flow for rapid diagnosis of leptospirosis. J. Med. Microbiol. **52:**897-901.

32. **Silva, M. V., E. D. Camargo, L. Batista, A. J. Vaz, A. P. Brandao, P. M. Nakamura, and J. M. Negrao.** 1995. Behaviour of specific IgM, IgG and IgA class antibodies in human leptospirosis during the acute phase of the disease and during convalescence. J. Trop. Med. Hyg. **98:**268-272.

33. **Smits, H. L., Y. V. Ananyina, A. Chereshsky, L. Dancel, A. F. R. F. Lai, H. D. Chee, P. N. Levett, T. Masuzawa, Y. Yanagihara, M. A. Muthusethupathi, E. J. Sanders, D. M. Sasaki, H. Domen, C. Yersin, T. Aye, S. L. Bragg, G. C. Gussenhoven, M. G. Goris, W. J. Terpstra, and R. A. Hartskeerl.** 1999. International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens. J. Clin. Microbiol. **37:**2904-2909.

34. **Smits, H. L., R. A. Hartskeerl, and W. J. Terpstra.** 2000. International multi-centre evaluation of a dipstick assay for human leptospirosis. Trop. Med. Int. Health **5:**124-128.

35. **Vijayachari, P., A. P. Sugunan, and S. C. Sehgal.** 2002. Evaluation of Lepto Dri Dot as a rapid test for the diagnosis of leptospirosis. Epidemiol. Infect. **129:**617-621.

36. **Wagenaar, J. F., T. H. Falke, N. V. Nam, T. Q. Binh, H. L. Smits, F. G. Cobelens, and P. J. de Vries.** 2004. Rapid serological assays for leptospirosis are of limited value in southern Vietnam. Ann. Trop. Med. Parasitol. **98:**843-850.

37. **Watt, G., K. Jongsakul, and C. Suttinont.** 2003. Possible scrub typhus coinfections in Thai agricultural workers hospitalized with leptospirosis. Am. J. Trop. Med. Hyg. **68:**89-91.

38. **World Health Organization.** 2003. Human leptospirosis: guidance for diagnosis, surveillance and control. World Health Organization, Malta.

39. **Wuthiekanun, V., N. Sirisukkarn, P. Daengsupa, P. Sakaraserane, A. Sangkakam, W. Chierakul, L. D. Smythe, M. L. Symonds, M. F. Dohnt, A. T. Slack, N. P. Day, and S. J. Peacock.** 2007. Clinical diagnosis and geographic distribution of leptospirosis, Thailand. Emerg. Infect. Dis. **13:**124-126.

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TABLE. Performance of four serological assays for leptospirosis in subjects groups from an urban center with high endemic transmission in Brazil.

Assay[a]	Sensitivity (95% CI)			Specificity (95% CI)
	Acute-phase (n = 96)	Acute-phase with <7 days of illness[b] (n = 72)	Convalescent-phase[c] (n = 50)	(n = 80)
MAT	68.8 (58.4 - 77.6)	45.8 (34.2 - 57.9)	100.0 (91.1 - 100.0)	100.0 (94.3 - 100.0)
ELISA BM	79.2 (69.4 - 86.5)	54.2 (42.1 - 65.8)	96.0 (85.1 - 99.3)	95.0 (87.0 - 98.4)
ELISA PB	87.5 (78.8 - 93.1)	66.7 (54.5 - 77.1)	92.0 (79.9 - 97.4)	87.5 (77.8 - 93.5)
DD	80.0 (70.3 - 87.2)	50.0 (37.9 - 62.1)	84.0 (70.3 - 92.4)	95.0 (87.0 - 98.4)
DS	72.3 (62.0 - 80.8)	32.9 (22.4 - 45.2)	80.0 (65.9 - 89.5)	100.0 (94.2 - 100.0)

278 [a] ELISA BM, EIE-IgM-Leptospirose (Bio-Manguinhos); ELISA PB, *Leptospira* IgM ELISA
279 (PanBio); DS, Dip-S-tick (PanBio); DD, LeptoTek Dri-Dot (Biomérieux); MAT, screening
280 criteria of ≥1:100 in the microagglutination test.

281 [b] Includes 29 patients from the acute-phase group and an additional 43 randomly-selected patients
282 for whom sera was obtained <7 days from the onset of illness.

283 [c] Randomly-selected from the 96 acute-phase patient group.

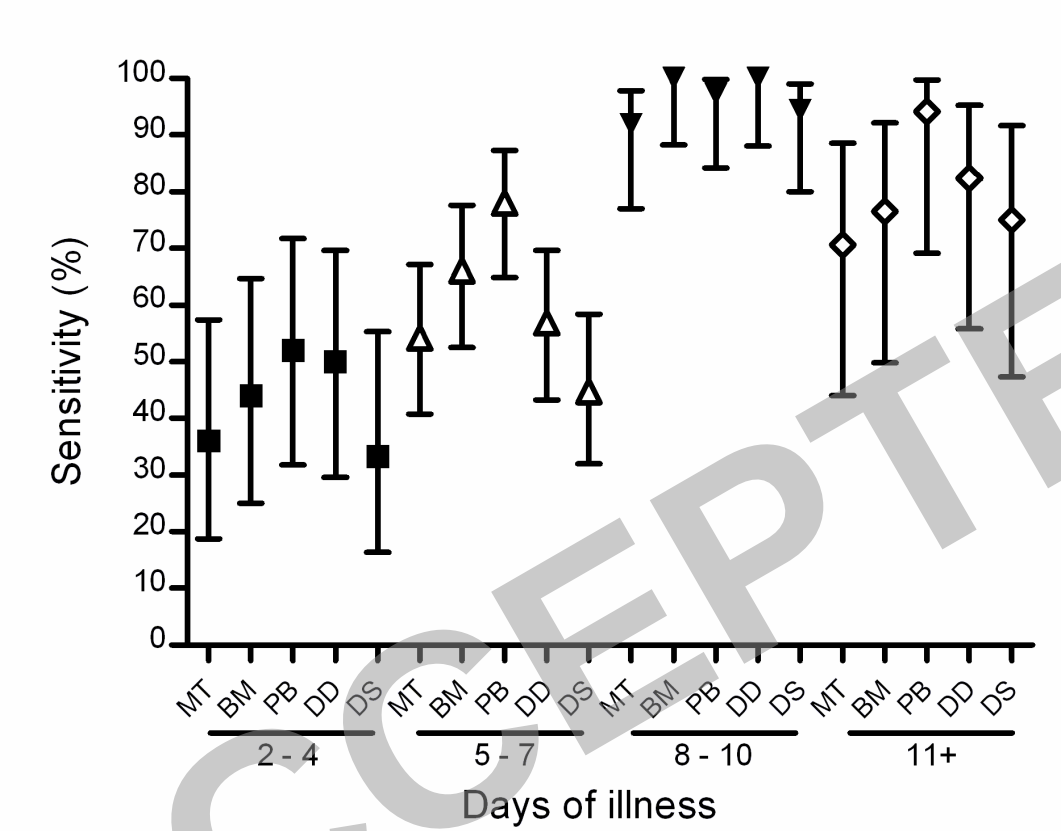

FIGURE. Sensitivity of the whole-cell *Leptospira* assays, according to days of illness. Mean sensitivity and 95% confidence intervals are shown for MAT screening criteria ≥1:100 (MT), EIE-IgM-Leptospirose (Bio-Manguinhos, BM), *Leptospira* IgM ELISA (PanBio, PB), Dri-Dot (Biomérieux, DD) and Dip-S-tick (PanBio, DS) assays.