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Establishment of Valid Laboratory Case Definition for Human Leptospirosis

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Abstract

Laboratory case definition of leptospirosis is scarcely defined by a solid evaluation that determines cut-off values in the tests that are applied. This study describes the process of determining optimal cut-off titers of laboratory tests for leptospirosis for a valid case definition of leptospirosis. In this case the tests are the microscopic agglutination test (MAT) and an in-house IgM enzyme-linked immunosorbent assay (ELISA) both on single serum and paired samples using a positive culture as the reference test in the Dutch population. The specificity was assessed using panels of sera from healthy donors, cases with known other diseases and non-leptospirosis cases with symptoms compatible with leptospirosis. Cases were divided into three periods corroborating the acute phase (1-10 days post onset of illness (DPO)), the early convalescent (11-20 DPO) and the late convalescent phase (>20 DPO). Cut-off titers for MAT and IgM ELISA were determined as 1:160 and 1:80 respectively for all three periods. These cut-off titers combined 100% specificity with a sensitivity that changed according to the stage of disease for both tests. The low sensitivities in the early acute phase are consistent with the dynamics of the humoral immune response. IgM ELISA yielded higher sensitivities compared to MAT in the acute and early convalescent stages. Moreover, the optimal sensitivity of MAT, the gold standard was < 82%, implying that a significant part of global cases is missed by this recommended test. MAT and IgM ELISA manifested partly complementary, resulting in a higher sensitivity when combining the results of these two tests. The availability of paired samples and of adequate clinical and epidemiological data are other parameters that will significantly increase the sensitivity of laboratory confirmation. This study enables fine-tuning of the current laboratory definition towards an improved case finding and implies that solid validation of laboratory parameters for case definition will improve both the diagnosis for individual patient care and for estimating the disease burden at a worldwide scale.

Keywords: Human leptospirosis; Case definition; Diagnostic methods; Serology; MAT; ELISA; Culture

Introduction

Leptospirosis, a zoonosis with a worldwide distribution, is an acute febrile illness caused by microorganisms of the genus *Leptospira* [1]. Leptospires enter the body through small cuts or abrasions, via mucous membranes such as the conjunctiva and possibly through wet skin and circulate in the blood stream, with a bacteremic phase lasting for up to 10 days post onset of the disease (DPO). The disease has a sudden onset with headache, fever, malaise, myalgia, conjunctival suffusion and sometimes a transient rash. Thereafter, the illness may rapidly develop into a severe, potentially fatal form with a high mortality rate. Because of the wide diversity of clinical signs, diagnosis of leptospirosis is difficult and depends upon a variety of laboratory assays such as detection of specific antibodies in the blood by microscopic agglutination test (MAT) and enzyme-linked immunosorbent assays (ELISA). Leptospires or their components may be detected in blood, urine or tissues by culture, dark field microscopy, immuno-staining or PCR [2,3]. The diagnosis of leptospirosis is not usually done on bacterial detection, but is mainly based on serology. Culturing provides proof of infection but is tedious and slow. This results in a high percentage of false negative findings, whereas the sensitivity of culturing usually does not exceed 20% [4–6]. The MAT is the reference standard in serodiagnosis. It is the most widely used diagnostic test for leptospirosis in the world and presents the only serological test that can be applied on any infected mammalian species. The MAT has the advantage of being specific for serogroups, although cross reactions and paradoxical reactions between serogroups do occur [7]. However, there are limitations in sensitivity in the early phase of the disease since detectable titers of antibodies appear in the blood about 5–10 days after the onset of disease [3], and sometimes later, especially if antibiotic treatment is instituted [8]. In addition, cross-agglutination of antibodies against other diseases and repeated exposure to *Leptospira* in endemic regions cause background titers [8,9]. The performance of the MAT is further complicated because live cultures of different *Leptospira* serovars prevalent in a particular geographical area are required for optimal performance [8]. Thus, the leptospirosis case definition including laboratory parameters varies in different epidemiological backgrounds and ideally requires local assessments of the laboratory tests. However, valid determination of cut-off titers for case definition is only scarcely done [9] or are not publicly presented [10,11] and current case definitions are mainly based on general assumptions and experience of experts [8,12,13]. In addition, case definitions formulate clinical manifestations either too wide or specify only forms of severe disease with signs and symptoms that often develop in the immune phase of the disease, when antibiotic treatment is less effective [14,15]. The lack of well defined laboratory criteria for regional case definitions is mainly due to a lacking knowledge

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on the assessment of optimal diagnostic accuracies of tests as well as on the easy availability of globally recommended criteria [8,12]. The global criterion for laboratory confirmation of a current *Leptospira* infection is usually defined as seroconversion or a four-fold rise in titer in paired serum samples or set at a single MAT titer ≥ 1:400 in the presence of clinical signs and appropriate history of animal contact [16]. This research describes the process of establishing optimal cut-off titers of tests for leptospirosis in order to obtain a valid laboratory case definition of leptospirosis, in this case the performance of MAT and ELISA in the population of The Netherlands. For this purpose, a positive culture is used as the reference test to define a case. For the clinical setting it is important to know how to interpret these diagnostic tests in relation to the time of sampling. Although cut-off titers will vary across distinct endemic situations, still this assessment will serve as a valuable example for others, irrespective the local situation. An improved, tailor-made case definition enables a better case finding and thus will contribute to an improved worldwide diagnosis of leptospirosis.

Materials and Methods

Patients and sera

Culture positive patients: In the period of investigation, the population in The Netherlands increased from 14.1 to 16.4 million with a median of 15.6 million. The WHO/FAO/OIE and National Collaborating Centre for Reference and Research on Leptospirosis (NRL) at KIT Biomedical Research in Amsterdam receives clinical material (whole blood, serum, EDTA blood, heparinized blood or urine) for testing from every patient suspected for leptospirosis in the Netherlands. Yearly, NRL receives about 600 specimens of suspected patients and confirms on average 30 cases [5,6]. Nowadays approximately 50% of the confirmed cases are imported infections, usually associated to adventurous activities such as white water rafting or jungle tracking during vacation in tropical countries [4,17]. Culture on blood is performed when sampled within 10 days after onset of symptoms. Culture on urine can be perfomed at any timepoint in the disease, allthough not often requested since the inoculation into the culture medium has to take place within two hours after voiding. From each blood sample routine diagnostics are performed by MAT and IgM ELISA. In the period 1980-2009 leptospirosis was confirmed by a positive culture in 107 patients, referred to as 'cases' in this paper. Samples were selected from these cases in order to have no more than one sample of each patient in each of the time periods 1 to 10 DPO, 11 to 20 DPO and more than 20 DPO according to the most relevant periods for laboratory confirmation. The first period is the acute phase defined by low sensitivity because of the absence of detectable amounts of antibodies in most samples. Antibody levels reach a maximum after about 10 days [18] and are well detectable in the early convalescent phase and steadily decrease after 20 days [3]. When multiple samples of one patient were present in a period of time, the sample with the lowest DPO was chosen (Table 1).

Healthy controls: Sera (n=110) from healthy blood bank donors were obtained from the local blood bank and served as healthy controls to determine cross-reacting antibodies in the population.

Controls with known disease to determine specificity based on selectivity: The following controls with known disease were used to screen for cross-reactivity to determine specificity based on selectivity. Sera from patients with human immunodeficiency virus (HIV) infection (n=20), hepatitis A virus infection (n=10), hepatitis B virus infection (n=9), malaria (n=20), toxoplasmosis (n=11) and meningococcal meningitis (n=20) since these infectious agents could cause symptoms similar to leptospirosis. Moreover, a control group with proven syphilis (n=19), also a spirochete and related to *Leptospira* and a group with autoimmune disease (rheumatoid arthritis, n=10; systemic lupus erythematosus, n=20) were used since these diseases are notorious for generating cross-reactive antibodies. These samples are referred to as 'known controls'.

Patients (n)	Samples (n) per patient	Sample taken from patients (n) at time period		
		DPO 1-10*	DPO 11-20	DPO >20
15	1	11**	0	4
58	2	26	26	
		29		29
			3	3
30	3	30	30	30

*Where 1-10 DPO is divided into 1-4 and 5-10 DPO the number of samples is 48 respectively 62.

** 5 of these patients have died therefore no follow up sample is available

Table 1: Samples selected according to disease period expressed as days post onset (DPO).

Controls with unknown disease: Sera were taken from patients suspected of leptospirosis but having a negative culture and for whom leptospirosis could not be confirmed. These samples are referred to as 'unknown controls' and were divided into an acute set, 1-10 DPO (n=62) and a convalescent set, >10 DPO (n=63).

Laboratory tests

Microscopic agglutination test: The microscopic agglutination test (MAT) was performed with a panel of live leptospires as described previously [19]. The panel consisted of 16 strains of the pathogenic serovars Bratislava, Ballum, Canicola, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Copenhageni, Poi, Pomona, Proechimys, Hardjo, Saxkoebing and Sejroe, and the non-pathogenic serovar Patoc. The sera from patients who were probably infected abroad were additionally examined by using a second panel of 12 strains representative of global leptospires, comprising the pathogenic serovars Australis, Rachmati, Bataviae, Celledoni, Cynopteri, Mini, Panama, Pyrogenes, Shermani and Tarassovi, and the non-pathogenic serovars Andamana and Semaranga. Non-pathogenic serovars were included to detect cross-agglutinating antibodies against pathogenic serovars that are not represented in the panels. Titers with these saprophytic serovars have no diagnostic relevance and are excluded from this assessment. Agglutination was performed in microtiter plates with serial twofold dilutions of the sera starting at 1:20 (5µl of serum, 45µl of phosphate buffered saline, and 50µl of antigen, incubated for 2-4 hours at 30°C). Agglutination was examined by dark-field microscopy. The titer was defined as the highest dilution giving 50% agglutination in comparison with that of the negative control [8,20].

IgM ELISA: The in-house developed ELISA for the detection of *Leptospira*-specific IgM antibodies (IgM ELISA) was performed with antigen prepared from a well grown culture of the local strain Wijnberg (serovar Copenhageni, serogroup Icterohaemorrhagiae) in EMJH medium [21]. The culture was killed by formalin (final concentration 0.5% v/v), heated in a boiling water bath for 30 min and centrifuged for 30 min at 10,000g. The supernatant was used for antigen. In each well of polystyrene micro titer plates (Greiner, Frickenhausen, Germany, Microlon 96 wells plates, medium binding) 100µl of the supernatant was left to evaporate at room temperature. The coated plates were

stored in a dry place at room temperature [20]. Before use, the plates were thoroughly washed with phosphate buffered saline (PBS) pH 7.2, containing 0.05% Tween 20 and serial twofold dilutions were made of the sera starting at 1:10 (10µl serum and 90µl of PBS containing 0.05% Tween 20 and 0.5% bovine serum albumin). After incubation with the serum dilutions for 1 h at 30°C the micro titer plates were washed and subsequently incubated with peroxidase-conjugated anti-human IgM (Biorad, Veenendaal, the Netherlands). After 1h incubation at 30°C, the plates were washed again and finally incubated with substrate (5-aminosalicylic acid; Merck). After 2 h the reaction was read at wavelength 492 nm. The titer was the last dilution giving an absorbance of more than half the value of a positive control serum [20].

Culture: The culture medium used was of Ellinghausen-McCullough as modified by Johnson and Harris (EMJH) supplemented with 5-fluorouracil (200µg/ml) (EMJH-FU) [8,16] rabbit serum (EMJH-RS) and 1% (V/V) fetal calf serum (EMJH-FCS) or combinations. Fletcher medium was prepared according to standard protocols [8,20]. The culture was done on blood (approximately 150µl) which was inoculated into the following culture media (6 ml): Fletcher medium, EMJH, EMJH-FU, EMJH-FCS-RS, and EMJH-FCS-RS-FU. Urine was diluted 1:10, 1:100 and 1:100 onto EMJH-FU and EMJH-FCS-RS-FU. Inoculated media are incubated for a maximum of 4 months at 30°C and biweekly checked for leptospiral growth by darkfield microscopy.

Data collection

From each sample Patient ID, sample ID, highest titer in MAT with the pathogenic strains, IgM ELISA titer and days post onset of symptoms (DPO) were retrieved from the KIT archives and entered into EPI INFO version 6.

Analyses

Sensitivity and corresponding confidence intervals was calculated for each cut-off titer by dividing the number of cases having at least this titer by the total number of cases. This was done for three time periods (1-10 DPO, 11-20 DPO and >20 DPO). Specificity and corresponding confidence intervals was calculated for three subgroups: healthy controls, known controls and unknown controls. The unknown controls were divided into two time points, 1-10 DPO and >10 DPO. Receiver Operating Characteristic (ROC) curves were constructed in Microsoft Excel. These curves show the true positive rate (in the cases) against the false positive rate (for each of the control subgroups) for the different cut-off titers of the MAT and IgM ELISA. For both tests for each time period (1-10 DPO, 11-20 DPO and >20 DPO) the cut-off values yielding an optimal combination of sensitivity and specificity were chosen. During the acute phase, detectable levels of anti-*Leptospira* antibodies are produced, so it can be expected that the sensitivity of MAT and IgM ELISA is much lower in the early acute phase (DPO <5). Therefore the acute phase was divided into 1 to 4 days and 5 to 10 days. From the optimal values combinations of MAT AND/OR IgM ELISA were made, and sensitivity and specificity were calculated on the complete dataset. ROC curves were plotted from these combinations in order to find out whether the sensitivity and/or specificity can be increased by combining the results from both tests. In the diagnosis of leptospirosis the optimal cut-off values are defined as those titers having the highest specificity. Confidence intervals will aid in determining which sensitivity and specificity are most precise based on the data. Results are presented based on MAT and IgM ELISA values on single sera, combinations of MAT AND/OR IgM ELISA values on single sera and seroconversion in paired sera. Serology on paired sera is much more reliable compared to a single sample [13] since a follow-up sample will take into account the effect of background or cross-reacting titers. Thus an optimal specificity is reached at a lower titer. We therefore argued that a one-step lower cut-off titer can be used for MAT and IgM ELISA on paired samples, herewith increasing the test sensitivity. Hence, the sensitivity is calculated on follow up samples using one-step lower cut-off titers in the tests. Paired samples from 68 cases were used to check the integrity of this approach.

Results

Patient samples

The total number of positive leptospirosis cases in the period 1980-2009 (based on culture and/or serology) is 919. One hundred and seven cases were found culture positive (11.6%). Leptospires were isolated from urine in 8 cases and from blood in 99 cases. Four cases were excluded from the analysis: first day of illness was not documented in three cases and ELISA results were missing in one case. The remaining group of 103 cases consisted of 95 men of whom 6 died (ranging from 6 to 30 DPO, median 7 DPO), and 8 women. Serovars belonging to the following serogroups were isolated: Icterohaemorrhagiae n=57, Grippotyphosa n=17, Autumnalis n=6, Celledoni n=3, Hebdomadis n=3, Pyrogenes n=3, Sejroe n=3, Bataviae n=2, Canicola n=2, Javanica n=2, Pomona n=2, Shermani n=2, Cynopteri n=1.

Determination of cut-off titers on single sera for MAT and IgM ELISA

The ROC curves showing the relationship between sensitivity and specificity according to each the acute phase and the early and late convalescent phases are depicted in Figure 1. The ROC curves include the most optimal sensitivity-specificity distribution for the set of healthy blood donors and indicate, as expected, that the specificity of MAT and IgM ELISA is not limited by cross-reacting antibodies in the healthy population. ROC curves for the known and unknown controls largely coincided, albeit that the IgM ELISA seemed to perform more stable than MAT. Specificity based on known controls (selectivity) indicates that cross-reacting antibodies to other diseases may interfere with the diagnosis and as such have importance that cannot be ignored. However, this selectivity is biased by inclusion of diseases that are unlikely to be confused with leptospirosis and overrates their impact on the practical situation that is better presented by the specificity based on unknown controls. Therefore, specificity based on unknown controls was selected as the most important parameter for further assessment of optimal cut-off values. From the ROC plots, MAT and IgM ELISA titers were selected for which optimal sensitivity and specificity was achieved for each time period (Table 2). When several optimal values were close together on the ROC curve all those values were listed in the table, the combination with the highest specificity being marked. For IgM ELISA a cut-off titer of ≥1:80 signified 100% specificity in all periods and was selected as the optimal cut-off value. This was associated with sensitivities of 25.0%, 91.7% and 70.8% for the time periods 1- 10 DPO, 11-20 DPO and >20 DPO, respectively. Cut-off titers of ≥1:40 did give a higher sensitivity in all categories but were associated with a lower specificity (Table 2). For MAT, 100% specificity was reached at a cut-off titer of ≥1:160. This was associated with a lower selectivity of 96.4% (not shown) and corresponded to sensitivities of 6.3%, 81.7% and 81.5%, for periods 1-10 DPO, 11-20 DPO and >20 DPO, respectively.

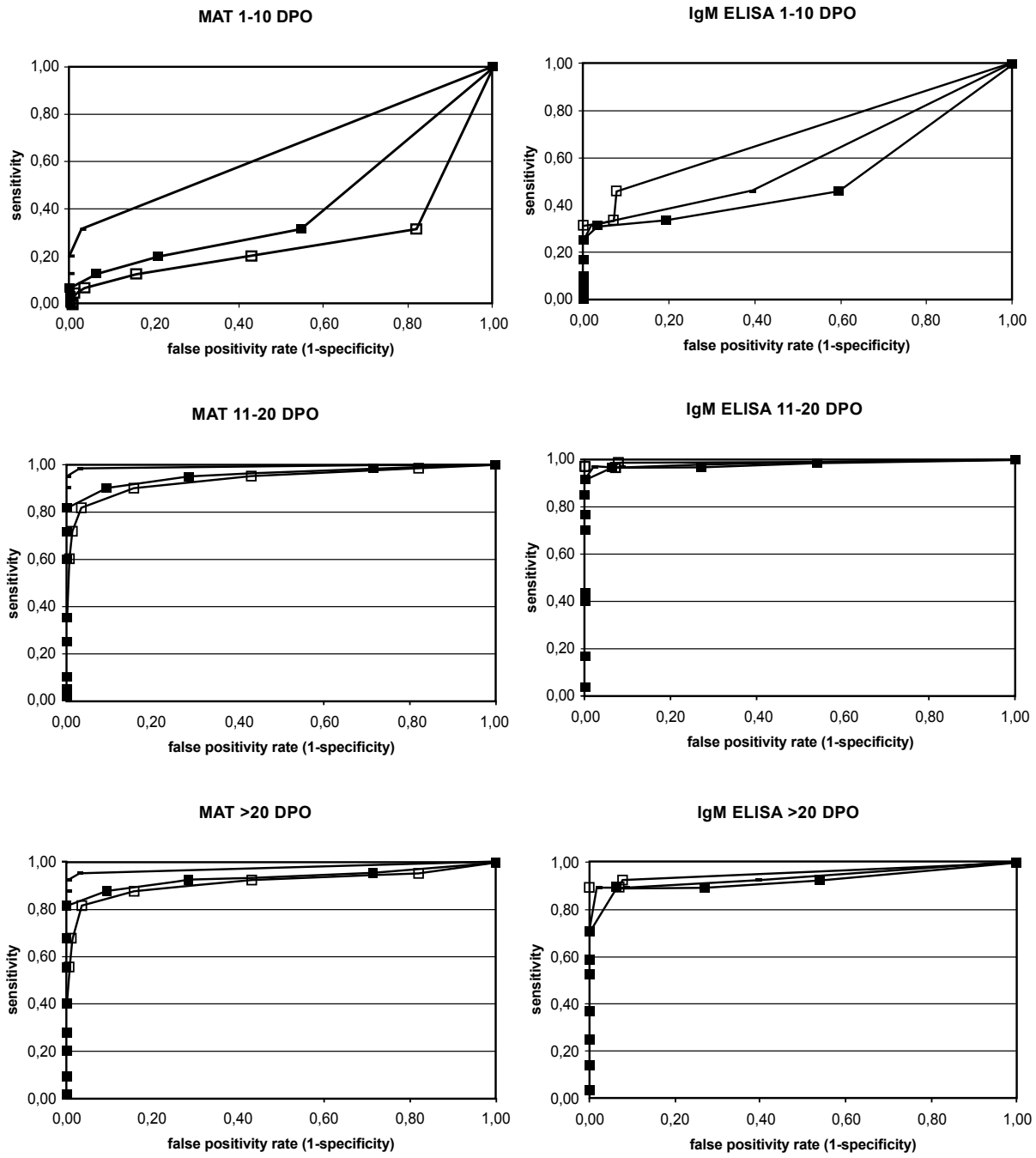

Figure 1: Receiver Operating Characteristic (ROC) Curve of cut-off titers of MAT and ELISA in different time periods healthy controls "_", known controls"□", unknown controls"■".

To investigate the acute phase, the time period 1-10 DPO was divided into two periods of 1-4 DPO (early acute) and 5-10 DPO (late acute). As expected, at the cut-off titer of ≥1:80, the sensitivity of IgM ELISA was only 6.3% in the early acute phase but rapidly increased to 53.2% in the late acute phase. For MAT this was 2.1 and 24.2%, respectively (Figure 2). This supports the notion that serology has minor importance at the very early phase of disease and only becomes useful during the second half of the first week of illness. The sensitivities of MAT in the acute phase are markedly lower than those of IgM ELISA, showing that IgM ELISA tends to give an earlier positive outcome compared to MAT.

Determination of cut-off titers on single sera for MAT combined with IgM ELISA: From the results in Table 2, sensitivities and specificities were calculated for all possible combinations of MAT and IgM ELISA in the samples and plotted in ROC curves (not shown). The optimal values of these combinations are shown in Table 3. Considering the limitations inflicted on MAT by the selectivity, the optimal cut-off titer of the MAT was again set to ≥1:160. For the categories DPO 1-10, both the cut-off titer combinations 'MAT ≥1:160 OR IgM ELISA ≥1:80' and 'MAT ≥1:160 AND IgM ELISA ≥1:80' did give 100% specificity but the sensitivity was much higher (25%) in the combination 'OR'. As indicated by the results for the separate tests,

DPO	Cut-off titer	MAT Sensitivity (%)	95% CI	MAT Specificity* (%)	95% CI	IgM ELISA Sensitivity (%)	95% CI	IgM ELISA Specificity* (%)	95% CI
1-10	≥1:40	19.8	13.1-28.9	79.0	67.4-87.3	31.3	22.9-41.1	96.8	89.0-99.1
	≥1:80	12.5	7.3-20.6	93.5	84.6-97.5	**25.0**	**17.4-34.5**	**100.0**	94.2-100
	≥1:160	**6.3**	**2.9-13.0**	**100.0**	94.2-100.0	16.7	10.5-25.4	100.0	94.2-100
11-20	≥1:40	95.0	86.3-98.3	71.4	59.3-81.1	96.7	88.6-99.1	**93.7**	84.8-97.5
	≥1:80	90.0	79.9-95.3	90.5	80.7-95.6	**91.7**	**81.9-96.4**	**100.0**	94.2-100.0
	≥1:160	**81.7**	**70.1-89.4**	**100.0**	94.2-100.0	85.0	73.9-91.9	100.0	94.2-100.0
>20	≥1:40	92.3	83.2-96.7	71.4	59.3-81.1	89.2	79.4-94.7	**93.7**	84.8-97.5
	≥1:80	87.7	77.5-93.6	90.5	80.7-95.6	**70.8**	**58.8-80.4**	**100.0**	94.2-100.0
	≥1:160	**81.5**	**70.4-89.1**	**100.0**	94.2-100.0	58.5	46.3-69.6	100.0	94.2-100.0

Bold text indicates the optimal cut-off for sensitivity and specificity based on the ROC plots with priority on a high specificity *specificity based on unknown controls

Table 2: Sensitivity and specificity and corresponding 95% confidence intervals (CI) of either MAT or IgM ELISA.

DPO	Cut-off titer MAT	AND/OR	Cut-off titer IgM ELISA	Sensitivity (%)	95% CI	Specificity (%)	95% CI
1-10	≥1:80	AND	≥1:40	10.4	5.8-18.1	100.0	94.2-100.0
	≥1:80	OR	≥1:40	33.3	24.7-43.2	90.3	80.4-95.5
	≥1:80	AND	≥1:80	10.4	5.8-18.1	100.0	94.2-100.0
	≥1:80	OR	≥1:80	27.1	19.2-36.7	93.5	84.6-97.5
	≥1:160	AND	≥1:40	6.3	2.9-13.0	100.0	94.2-100.0
	≥1:160	OR	≥1:40	31.3	22.9-41.1	96.8	89.0-99.1
	≥1:160	AND	≥1:80	6.3	2.9-13.0	100.0	94.2-100.0
	≥1:160	**OR**	**≥1:80**	**25.0**	**17.4-34.5**	**100.0**	**94.2-100.0**
11-20	≥1:80	AND	≥1:40	90.0	79.9-95.3	98.4	91.5-99.7
	≥1:80	OR	≥1:40	96.7	88.6-99.1	85.7	75.0-92.3
	≥1:80	AND	≥1:80	86.7	75.8-93.1	100.0	94.2-100.0
	≥1:80	OR	≥1:80	95.0	86.3-98.3	90.5	80.7-95.6
	≥1:160	AND	≥1:40	81.7	70.1-89.4	100.0	94.2-100.0
	≥1:160	OR	≥1:40	96.7	88.6-99.1	93.7	84.8-97.5
	≥1:160	AND	≥1:80	80.0	68.2-88.2	100.0	94.2-100.0
	≥1:160	**OR**	**≥1:80**	**93.3**	**84.1-97.4**	**100.0**	**94.2-100.0**
>20	≥1:80	AND	≥1:40	81.5	70.4-89.1	98.4	91.5-99.7
	≥1:80	OR	≥1:40	95.4	87.3-98.4	85.7	75.0-92.3
	≥1:80	AND	≥1:80	67.7	55.6-77.8	100.0	94.2-100.0
	≥1:80	OR	≥1:80	90.8	81.3-95.7	90.5	80.7-95.6
	≥1:160	AND	≥1:40	76.9	65.4-85.5	100.0	94.2-100.0
	≥1:160	OR	≥1:40	93.8	85.2-97.6	93.7	84.8-97.5
	≥1:160	AND	≥1:80	63.1	50.9-73.8	100.0	94.2-100.0
	≥1:160	**OR**	**≥1:80**	**89.2**	**79.4-94.7**	**100.0**	**94.2-100.0**

Bold text indicates the optimal cut-off for sensitivity and specificity combinations with priority on a high specificity.

Table 3: Optimal sensitivity and specificity by combining MAT and IgM ELISA results.

serology on early acute sera samples is not very helpful, also when combining the titers of the two tests (Figure 2). However, for DPO 5-10, the cut- off titer combination 'MAT ≥1:160 OR IgM ELISA ≥1:80' represented a sensitivity of 53.2% with a specificity of 100% (Figure 2). In the period 1-10 DPO, all samples having a MAT titer ≥1:160 also had an IgM ELISA titer of ≥1:80, so the sensitivity of 'MAT ≥1:160 OR IgM ELISA ≥1:80' is determined by the IgM ELISA. The optimal combination for both the periods 11-20 DPO and >20 DPO was 'MAT ≥1:160 OR IgM ELISA ≥1:80'. Specificity was in both periods 100% and associated with a decrease of sensitivity from 93.3 to 89.2% in the later stage, reflecting the decrease of antibody levels in the late convalescent stage of disease [3].

Paired samples

Sensitivity for follow up samples or samples that are accompanied by clinical and epidemiological data compatible with leptospirosis was calculated for a cut-off titer 1:40 for IgM ELISA and 1:80 for the MAT, accounting both for seroconversion and a minimal 4-fold titer rise in both tests. For the time period 1-10 DPO, this implied a sensitivity of 12.5 and 31.3% for MAT and IgM ELISA, respectively (Table 2). For the category 11-20 DPO this was 90.0% and 96.7% respectively, declining to 87.7%, and 89.2% for >20 DPO. When combining the MAT and IgM ELISA results at these cut-off titers, the three categories had sensitivities of 33.3, 96.7 and 95.4%, respectively (Table 3). In our study, 68 paired samples with initial titers not fulfilling the cut-off titer combination 'MAT ≥1:160 OR IgM ELISA ≥1:80', showed in 67 cases (98.5%) in the follow up sample seroconversion or at least a four-fold titer rise after 2 to 121 days (median 12 days). Sixty three cases did show seroconversion or titer rise in the follow up sample exceeding

the criteria set for a single sample, while another four did meet the criteria set for paired samples. The case not showing seroconversion was infected with serovar Grippotyphosa; samples were taken on 3 and 30 DPO.

Discussion

Together with the difficult diagnosis of leptospirosis both in the clinic and in the laboratory, inadequate case definitions probably form a major cause for the global underestimation and unawareness of the disease. Good knowledge on how to assess optimal accuracy of laboratory tests is mostly lacking and general recommended criteria, rather than tailor-made ones, are adopted. In this retrospective case control study, the optimal cut-off values for leptospirosis confirmation by MAT and IgM ELISA in The Netherlands are determined, using positive culturing results as the reference test. Taking into account the dynamics of the humoral response [3], single samples were analysed according to three time periods, i.e. the acute stage of illness (1-10 DPO), the early convalescent stage (11-20 DPO) and the late convalescent stage (>20 DPO). Cut-off titers of 1:80 and 1:160 for IgM ELISA and MAT were determined for all three periods. This was associated with a specificity of 100% for both tests. However, the sensitivities varied markedly from 6.3 to 81.7% for MAT and 25.0 to 91.7% for IgM ELISA in the acute and early convalescent phase. Thus, sensitivities of MAT and IgM ELISA are low at the acute stage of illness and require a follow-up sample for confirming seroconversion or a significant titer rise. To assess the impact of the increasing immune response on the diagnostic outcome in more detail, the acute phase was split in an early (1-4 DPO) and late (5-10 DPO) stage. Rapid increases in sensitivity in both tests across the two stages were noticed; i.e. the sensitivity for MAT rose from 2.1 to 24.2% and the sensitivity of the IgM ELISA from 6.3% to 53.2%. Moreover, testing paired samples markedly increased the sensitivity of the diagnosis. This justifies two conclusions. (i) These findings support the notion that serology has minor importance at the very early phase of disease. (ii) These results underline the importance of using a follow up sample for confirmation by serology. However, unlike generally stated that paired samples should preferably be collected two weeks apart [13], much shorter intervals appear relevant. Seroconversion was observed in samples taken as few as two days apart. Therefore, we advocate the use of repeated samples, albeit at intervals of one week or shorter in case of urgent suspicion. The MAT is the reference test in serodiagnosis of leptospirosis and is often referred to as the gold standard. This nomination is not supported by this study. MAT was at the best 81.7% sensitive in the convalescent stage, implying that 2 of 10 cases are missed when only MAT is used on a single sample. The IgM ELISA did perform much better (sensitivity 91.7%) and was significantly more sensitive compared to MAT in the late acute stage of illness. It is therefore tempting to designate IgM ELISA as the new gold standard. There are, however, caveats. This study was performed on Dutch cases with low infection backgrounds and concerned an in-house ELISA. This ELISA is based on the determination of a titer from serial dilutions [19,22]. This approach is probably more robust than using single well platforms and, hence, our results cannot be translated to these other formats. Moreover, high prevalence countries might have higher background titers, requiring different cut-off titers of both MAT and ELISA that might favour the use of MAT. Additionally, unlike MAT, ELISA is a genus specific tests that does not enable the assessment of presumptive infecting serogroups, and as such lacks an important epidemiological application. In The Netherlands, MAT is always

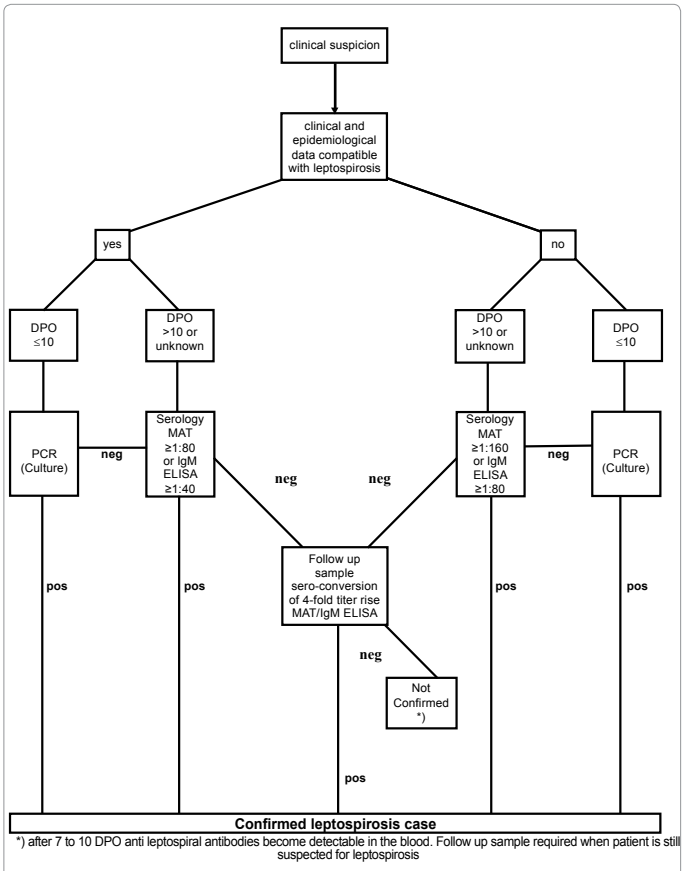

Figure 3: Algorithm, assisting with interpretations and conclusions on the outcome of laboratory testing.

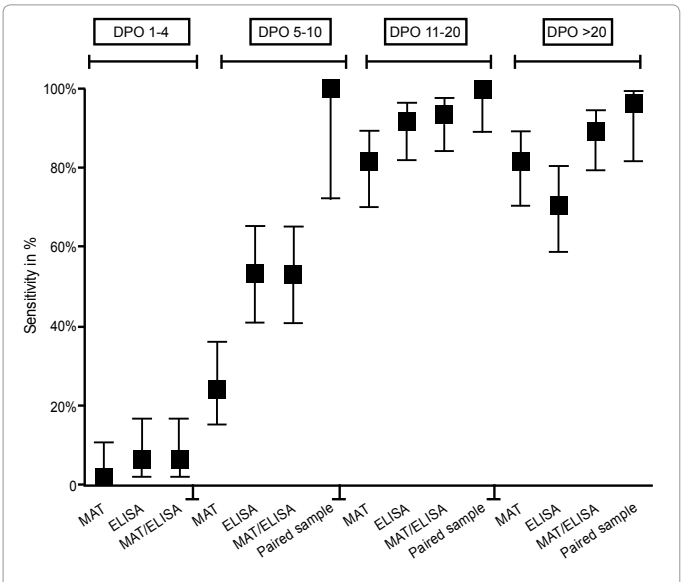

Figure 2: Sensitivity and corresponding 95% CI in the periods 1-4 DPO, 5-10 DPO, 11-20 DPO and >20 DPO of MAT (cut-off titer ≥1:160), IgM ELISA (cut-off titer ≥1:80), combination "MAT ≥1:160 OR IgM ELISA ≥1:80" and 'paired samples' of which follow up sample is taken in the designated time period. This category of 'paired samples' include single samples that are supported by epidemiological and clinical data compatible with leptospirosis.

performed along with IgM ELISA. Therefore the impact of diagnostic value by combining the results of both tests was investigated. Combination of tests had no effect on the acute phase of the disease that was dominated by the sensitivity of the IgM ELISA but resulted in a markedly increased sensitivity in the early convalescent stage. Applying cut-off titers of 1:160 and 1:80 for MAT and IgM ELISA, a sensitivity of 93.3% was reached by combining MAT 'OR' IgM ELISA results, comparing to 81.7 and 91.7%, for each test, respectively. Apparently, the two tests are partly complementary at this stage of illness. This observation substantiates the importance of using two distinct tests. This study unambiguously demonstrates that serology largely fails in the acute phase of the disease. This period is of most clinical importance because adequate antibiotic treatment has to be initiated as early in the illness as possible [14,15]. Thus, serology is not very helpful in an early stage of leptospirosis when confirmation is most required. Late confirmation by serological testing, presents a major obstacle in clinical decision making. Clinicians have to rely on their own judgement based on mostly uncharacteristic symptoms to initiate appropriate (antibiotic) treatment. On one hand, low titers during early illness are highly unreliable because of a low accompanying specificity (Table 2). On the other hand, such low titers might be a sign of a developing leptospirosis and as such, should not be ignored. We deal with this contradiction by informing the consulting clinician promptly upon finding low reactions and explain their significance. Following this strategy, the clinician is provided with early indications on a potential *Leptospira* infection that support decisions on the initiation or pursuit of a suitable treatment. Fortunately, nowadays validated real time PCRs are available [23,24]. These PCRs have a high diagnostic accuracy notably in this early acute phase, hence perfectly complementing serodiagnostic testing. We are aware that a lack of experience and financial potential might hamper the introduction of real time PCR notably in those regions where the disease is highly prevalent but, nevertheless, strongly recommend its implementation in the diagnostic services. In absence of real time PCR, laboratory testing will largely benefit from well established criteria that define positive outcomes of serological testing. The study described in this paper serves as a template for other such studies in distinct epidemiogical settings. Such local evaluation is of great importance because different cut-off titers in the MAT have been demonstrated in high compared to low endemic situations [9]. In our study, relatively low cut-off titers for MAT were defined, i.e., ≥1:160 for single samples and ≥1:80 for paired samples. There are two reasons for these low cut-off titers: (i) The infection background in The Netherlands is low and as such impacts to a lesser extent on the specificity. This also counts for the IgM ELISA. (ii) Historically, at NRL *Leptospira* strains are not diluted before performing the MAT as recommended [8] but full grown cultures are used. A higher density of *Leptospira* strains will reduce the titer by one dilution step at the minimum [16]. Consistently, in the MAT proficiency testing, we find our titers below the median titer [25]. Cut-off titers are based on single serum samples, without information on adequate clinical and epidemiological findings. It is common notice that knowledge of clinical and epidemiological data are highly valuable and should largely contribute to the confirmation of suspected leptospirosis [8,26]. For the same, testing of paired serum samples is strongly recommended and case definitions use lower cut-off titers on paired samples than on single ones [8,12]. In line with this argument, we applied cut-off titers that were one dilution step lower than those deduced for single samples. When testing this approach on a set of 68 paired samples, all but one fulfilled the criteria of these lower cut-off titers. The one exception was an infection with Grippotyphosa that did not give any detectable antibody response. Grippotyphosa infections are notorious for causing transient or delayed immune responses that might be missed [3,8,11]. Our evaluation allowed us to change our current and more stringent case definition based on preliminary data analysis on single serum samples [27] and consequently is expected to improve the case finding in The Netherlands. The use of different cut-off values in different epidemiological situations might lead to confusion. Therefore, we have designed an algorithm, assisting with interpretations and conclusions on the outcome of our laboratory testing (Figure 3). This algorithm includes application of a validated real time PCR [23]. We anticipate that the assessment of our case definition as well as the algorithm presented in this paper is not only useful for application in The Netherlands but can easily be adapted into a useful assessment tool in different epidemiological backgrounds. As a concluding remark we mention that a lack of notification is not the only reason for the underestimation of leptospirosis [28]. As we have shown, inadequate case definition and a questionable sensitivity of the reference MAT both are also causes of missing cases.

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