Evaluating reagents for serological diagnosis of Leptospirosis EPINET 1 Workshop (Guam, December 2001)

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1. Introduction

During the EPINET 1 Workshop (Guam, December 2001), it was agreed that most outlying laboratories (i.e. Level 1) of the Pacific Public Health Surveillance Network (PPHSN) should all have access to rapid tests for the diagnosis of leptospirosis. Given the sub-optimal performances of these reagents, all positive or negative tests in suspicious clinical or epidemiological contexts would then have to be systematically confirmed by a so-called Level 2 laboratory using more reliable operating protocols (in particular, ELISA microplate assay) or reference techniques.

In order to ensure regular and rapid supply to Pacific island countries, it seemed best to use PanBio (Brisbane, Australia) products. Some of these products have already undergone international evaluations (Leptospira – Dip-S-Ticks, in particular [1,2]) and are available in several countries in the Pacific region.
It was, then, possible to propose the following kits for leptospirosis survey:

- *Rapid single test for Level 1 laboratories:*
  - Dip-S-Ticks IgM Leptospira, PanBio reference 5065M-02-10 or 5065M-01-50 (10 or 50 tests)
- *Confirmation test for Level 2 laboratories (Guam, Noumea, Papeete and Suva):*
  - Leptospirosis: ELISA IgM microwell kit, PanBio reference LPM-200 (96 tests)

At the current stage of implementation of the final technical recommendations of EPINET 1, a Level 2 laboratory with adequate experience in leptospirosis testing should use these reagents under real conditions for a limited period of time. In fact, without attempting a complete re-evaluation, it was important for a Level 2 laboratory in the region to:

- master handling of these products so as to provide technical assistance, if necessary, to other laboratories in the network,
- verify the reported performances, in terms of sensitivity and specificity.

This work, which was limited in scope, was conducted by the New Caledonia Pasteur Institute (IPNC) in August 2002.

2. Materials and methods

2.1 Evaluation principle:

Using samples from the serum bank of IPNC, two panels of samples which had initially tested positive for leptospirosis or other diseases that could constitute differential diagnoses were simultaneously analysed with PanBio rapid test and microplate technique, by the thermoresistant antigen (TA) macro-agglutination technique and then again by the conventional technique used at IPNC, taken as reference, i.e.:

- the micro-agglutination technique (MAT) as per Martin and Petit [3], implemented against a battery of 9 *Leptospira* serogroups endemic to New Caledonia (9 pathogenic serovars from the species *L. interrogans* : *Australis australis, Ballum ballum, Bataviae bataviae, Canicola canicola, Icterohaemorragiae icterohaemorragiae, Icterohaemorragiae copenhageni, Panama panama, Pomona pomona, Pyrogenes pyrogenes)* and one saprophyte strain : *L. biflexa Seramanga Patoc.*

Each result obtained from an evaluated kit was identified as a true positive (TP) or a true negative (TN), if it matched the result obtained using the reference technique. Otherwise, it was considered a false positive (FP) or a false negative (FN).

The total number (Σ) of each type of result obtained (ΣTP, ΣTN, ΣFP, ΣFN) made it possible to calculate the following characteristics [4]:

- Sensitivity (%): ΣTPx100 / ΣTP + ΣFN
- Specificity (%):ΣTNx100 / ΣTN + ΣFP
- Positive predictive value (%): ΣTPx100 / ΣTP + ΣFP
- Negative predictive value (%):ΣTNx100 / ΣTN + ΣFN

NB: Given the small number of samples included in the study panels, the statistical values of the parameters above were not calculated.

2.2 Biological material used

2.2.1 Sensitivity panel:

20 positive sera from 2001 and 2002 (highest MAT titre equal to or higher than 200), including:
- 7 positive for serogroup *Icterohaemorragiae* (the highest titre),
- 3 positive for serogroup *Australis* (the highest titre),
- 1 positive for serogroup *Panama* (the highest titre),
- 3 positive for serogroup *Canicola* (the highest titre),
- 3 positive for serogroup *Ballum* (the highest titre),
- 3 positive for serogroup *Seramanga Patoc* (the highest titre),

9 negative sera from 2002 (the highest MAT titre equal to or lower than 100), including:
- 5 sera also negative for dengue fever,
- 4 clinically suspect sera, corresponding to early samples from patients whose illnesses were subsequently confirmed.

These 29 samples included 4 seroconversion panels (1 with 3 sequential samples and 3 patients with a pair of sera, i.e. early and late).

2.2.2 Specificity panel

This comprised 25 sera reactive to diseases frequently encountered in the Pacific region, documented as follows:
- 4 positive Hepatitis A IgM sera
- 2 positive Hepatitis B (anti-HBc) IgM sera
- 2 positive anti CMV IgM sera,
- 2 positive anti *Toxoplasma gondii* IgM sera
- 2 positive syphilis serology sera (simultaneously positive for TPHA and VDRL),
- 2 positive HIV serology sera (2 mixed ELISA HIV1/2),
- 2 positive influenza serology sera (with titre 1/40 or higher for Complement Fixation antibodies),
- 5 positive dengue fever IgM sera,
- 4 positive rheumatoid factor sera (simultaneously positive for Waaler-Rose and latex tests).

2.3 Evaluated products

- Dip-S-Ticks IgM Leptospira (PanBio reference 5065M-01-50), semi-quantitative dipstick test (Dot-blot), conducted at 50° C, in about 30 minutes,
- Leptospira IgM Capture (PanBio reference LPM-200): ready-to-use ELISA microwell test, conducted in about 1½ hours.
- TR antigen (Bio Rad reference 79623): macroscopic agglutination test of a heated suspension of *Leptospira biflexa Patoc*, which can be carried out in 5 minutes.

3. Results and analysis

The overall characteristics of the evaluated products are given in Table I, analysis of the cross-reactions in Table II, and study of the seroconversion panels in Table III.

3.1 Dip-S-Ticks Leptospira rapid text (PanBio)

Fairly easy to use, this test does, nevertheless, require a minimum amount of technical material (precision pipette, water bath or thermostatically controlled 50° heating block). It can, then, be used on sites with limited equipment but cannot be conducted outside a laboratory. It is easy to read the results which have judiciously been made quantifiable by a series of 4 increasing antigen solution spots (guide of 0 to 4 arbitrary units, with the result considered positive at 2 or more). After testing, the dried strips can be kept for later readings, as the colours appear to remain stable for a long time (more than a month in our experience to date).

The specificity of the response is excellent (100%), since no cross reactions were uncovered with the non-leptospirosis disease panels. This results matches an evaluation conducted recently in Hawaii which gave a specificity of 95% [2].

Sensitivity was evaluated at 80%. Four samples which had been positive in the micro-agglutination test gave a response which was zero or less than 2 with the Dip-S-Ticks: 1 sample that was positive at $1/400^{th}$ for the Panama serogroup, another at $1/12800^{th}$ for the *Icterohaemorragiae copenhageni* serovar and 2 sequential samples from a single patient showing a seroconversion for the *Australis* serogroup (score on the late sample at 1.5, whereas the MAT is positive at $1/3200^{th}$). The other three seroconversion panels were correctly identified. It was noted that there was a reaction, sometimes strong, at the beginning of the infection at the time when the MAT shows coagglutination for several serogroups, including non-pathogens such as *Patoc* (see Table III, patient GR).

The negative predictive value of this reagent was not 100%, but just 89.5%. When used as a front-line screening test, all negative tests in spite of a clinical context resembling leptospirosis, would have to be retested by another technique - especially if the sample was taken soon after the symptoms first appeared. In Smits *et al*'s evaluation (1999 [1]) of a first generation Dipstick, they found sensitivity to be 61% for early samples (illness of less than 10 days) and 87.4% for the latest samples.

3.2 TR antigen macro-agglutination test

This was one of the first tests put out for serological screening of leptospirosis. It is very simple and very rapid to use; however, the agglutinations obtained are often difficult to read. Although still on the market, it has been widely criticised for its lack of specificity and sensitivity, which prohibits its use as the sole screening test [3]. The results obtained during our evaluation confirmed these poor performances. Using all the samples tested, the sensitivity of the macro-agglutination test was calculated to be 45%, its specificity 76.5%, leading to a negative predictive value of only 70.3%. Under these conditions, this test cannot be recommended.

3.3 ELISA microplate test (PanBio Leptospira IgM Capture):

This product is simple to use as all the components are delivered in a ready-to-use form. The stability of reagents after the tubes have been opened make it possible to conduct small series (e.g., 6 series of 11 samples for a kit of 96 tests). However, the technique does require specific equipment: incubator, microplate washer and reader, precision pipettes. For that reason, it can only be conducted in an experienced laboratory by adequately trained staff.

Sensitivity was measured at 90%. Of the 20 sera which tested positive with the MAT, two gave negative responses with the microplate ELISA test: 1 sample that was positive at $1/400^{th}$ for the Panama serogroup and another at $1/12800^{th}$ for the *Icterohaemorragiae copenhageni* serovar. It should be noted that both of these samples also gave negative responses to the Dip-S-Tick rapid test. This probably involved specific characteristics of the strains, rather than the serogroup (this should be checked for *Panama*, however), with regards to the antigens of the PanBio reagents. In Zockowski *et al*'s evaluation (United Kingdom, 2001), the sensitivity of this reagent as compared to the MAT was equivalent (90%) [5], whereas the Australian study of Winslow *et al* reached a conclusion of 100% ; the authors even noted an earlier positive response from ELISA than from the MAT during analysis of sequential samples [6]. The four seroconversion panels studied showed responses in line with the MAT, with, however, as with the rapid test, a strong reactivity to the *Patoc* serovar antibodies which appear during coagglutination episodes found in the early phase of the illness.

Specificity was evaluated at 97.1%, with a single cross reaction noted for a sample that had high reactivity to the rheumatoid factor. This interference has also been described by Smits *et al* for a rapid strip test [7]. The positive predictive value of the results was estimated at 94.7%. In the Hawaiian study mentioned above [2], specificity and PPV of this kit were respectively 98 and 86%.
Use of this test to confirm a screening technique such as the Dip-S-Tick does, then, seem, at least in part, appropriate.

4. Conclusion

Several types of rapid single test reagents were proposed for screening for leptospirosis, few of which combine the optimum characteristics of sensitivity and specificity [2]. The performances measured for the PanBio Dip-S-Tick during our work were close to those of other recent studies: it was found to have an excellent specificity (100%) but only a mediocre sensitivity (80%) in comparison to the MAT test. In the view of an acceptable compromise, it is possible to use the Dip-S-Tick for screening, especially as there are no specific difficulties in conducting this test and it is easy to read. However, it must be pointed out that negative results in a suspicious clinical and biological context must absolutely be reconfirmed by another, more sensitive method and should lead to repeating the test on a later sample.

With its ease of use and a sensitivity level evaluated at some 90%, the IgM Capture test can be used as a confirmation method. However, it is still important to use the micro-agglutination test to study any epidemiologically or clinically suspect samples which test negative, along with the positive ones in order to be able to determine the involved serogroup. This last information cannot be obtained by other serological techniques and is of significant epidemiological interest for the investigation into the mode of contamination and leptospirosis control at the community level. The MAT also makes it possible to exclude leptospirosis in the event of reactivity limited to non-pathogenic serovars.

Our evaluation confirmed that the macro-agglutination test, the oldest screening technique, has performances which are inadequate for use as a reliable field test. For that reason, its use is inadvisable.

The two PanBio products evaluated, which are easy to procure in the Pacific, can, then, be recommended to Level 1 & 2 Pacific Public Health Surveillance Network (PPHSN) laboratories for the serodiagnosis of leptospirosis. With a view to better describing the still poorly known regional epidemiology of this disease, it would be interesting to have all positive samples reconfirmed by the micro-agglutination technique.

5. References

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Panel	Kit evaluated: Name	Kit evaluated: Kit Result	Reference method : MAT (a): Number	Reference method : MAT (a): Positive	Reference method : MAT (a): Negative	Performances of Kit evaluated: Sensitivity	Performances of Kit evaluated: PPV (b)	Performances of Kit evaluated: Specificity	Performances of Kit evaluated: NPV (c)
Specificity	Lepto Dip-S-Tick (PanBio)	Positive (2 – 4 UA (d))	0	0	0	-	-	100%	100%
		Negative (< 2 UA)	25	0	25				
	Leptospira IgM Capture (PanBio)	Positive	1	0	1	-	-	96%	100%
		Negative	24	0	24				
	TR Agglutination	Positive	4	0	4	-	-	84%	100%
		Negative	21	0	21				
Sensitivity	Lepto Dip-S-Tick (PanBio)	Positive (2 – 4 UA)	16	16	0	80%	100%	100%	69.2%
		Negative (< 2 UA)	13	4	9				
	Leptospira IgM Capture (PanBio)	Positive	18	18	0	90%	100%	100%	81.8%
		Negative	11	2	9				
	TR Agglutination (Bio Rad)	Positive	13	9	4	45%	69.2%	55.6%	31.3%
		Negative	16	11	5				
Total	Dip-S-Tick Lepto (PanBio)	Positive	16	16	0	**80%**	**100%**	**100%**	**89.5%**
		Negative	38	4	34				
	IgM Lepto (PanBio)	Positive	19	18	1	**90%**	**94.7%**	**97.1%**	**94.3%**
		Negative	35	2	33				
	TR Agglutination	Positive	17	9	8	**45%**	**52.9%**	**76.5%**	**70.3%**
		Negative	37	11	26				

(a) : micro agglutination test, positivity threshold : 1/200, (b) : positive predictive value, (c) : negative predictive value, (d): arbitrary units

Table I : Specificity, sensitivity, positive and negative predictive values of the tests evaluated for the diagnosis of leptospirosis, in comparison to the New Caledonia Pasteur Institute reference technique (micro agglutination test MAT).

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12/12/02

Panel Composition:			Reference Method		Kits evaluated					
			MAT (IPNC)		Lepto Dip-S-Tick (PanBio)		Lepto IgM Capture (PanBio)		TR Agglutination (Biorad)	
Pathology	Parameter	number	Positive	Negative	Positive	Specificity	Positive	Specificity	Positive	Specificity
Hepatitis A	IgM	4	0	4	0	100.0%	0	100.0%	1	75.0%
Hepatitis B	HBc IgM	2	0	2	0	100.0%	0	100.0%	0	100.0%
CMV	IgM	2	0	2	0	100.0%	0	100.0%	1	50.0%
Toxoplasmosis	IgM	2	0	2	0	100.0%	0	100.0%	0	100.0%
Syphillis	TPHA & VDRL	2	0	2	0	100.0%	0	100.0%	0	100.0%
HIV	Total Ab	2	0	2	0	100.0%	0	100.0%	0	100.0%
Influenza	Total Ab	2	0	2	0	100.0%	0	100.0%	0	100.0%
Auto-immune disease	Rheumatoid Factor	4	0	4	0	100.0%	1	75.0%	0	100.0%
Dengue	IgM	5	0	5	0	100.0%	0	100.0%	2	60.0%
Total non-leptospirosis pathologies		25	0	25	0	**100.0%**	1	**96.0%**	4	**84.0%**

Table II : Specificity of the tests evaluated for the diagnosis of leptospirosis, in comparison to the New Caledonia Pasteur Institute's reference technique (micro agglutination test MAT) with regards to samples which tested positive for other diseases.

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Patient ID.	Sample and timing (days)		MAT (micro-agglutination test) Highest titre (thresh. : 200)	MAT (micro-agglutination test) Serogroup	Kits evaluated Dip-S-Ticks (Arbitrary units: thresh. 2)	Kits evaluated ELISA IgM Capture OD (a) patient/thresh. value	Kits evaluated TR Antigen (macro-agglutination)
GR	Early	0	200	*Patoc*	3	3.23	**Negative**
	Late 1	2	400	*Patoc*	3	2.05	Positive
	Late 2	16	1600	*Icterohaemorrhagiae*	3	1.41	Positive
GE	Early	0	200	*Patoc*	**0**	1.1	Positive
	Late	14	3200	*Australis*	**1.5**	1.51	**Negative**
LA	Early	0	50	*Icterohaemorrhagiae*	0	0.13	**Positive**
	Late	40	800	*Icterohaemorrhagiae*	2.5	1.05	**Negative**
KA	Early	0	100	*Icterohaemorrhagiae*	0	0.31	**Positive**
	Late	12	12800	*Icterohaemorrhagiae*	2	1.59	Positive

(a) OD: Optical Density

Table III : Sensitivity of evaluated tests used for the diagnosis of leptospirosis in comparison to the New Caledonia Pasteur Institute's reference technique (micro agglutination test MAT), for seroconversion panels.

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12/12/02