THE NEW MICROBIOLOGICA, 27, 149-154, 2004

EVALUATION OF AN IGM-ELISA TEST FOR THE DIAGNOSIS OF HUMAN LEPTOSPIROSIS

G. Vitale, C. La Russa, A. Galioto, N. Chifari, C. Mocciaro, R. Caruso, A. Micalizzi, P. Mansueto, S. Di Rosa[1], S. Mansueto

Department of Clinical Medicine and Emerging Pathologies and Regional Centre of Reference for Leptospirosis, University of Palermo, Italy
[1]Azienda Ospedaliera "Villa Sofia - C.T.O.", Palermo, Italy

SUMMARY

Leptospirosis is a zoonosis with a worldwide distribution very common in most countries. In Italy this acute febrile illness is more frequent in the Northern than in the Southern regions. In the period 1994-1996, the number of cases of Leptospirosis in Sicily was lower with respect to the northern-central regions (7.2% and 73.4% respectively).
Between January 1990 and December 1999, a total of 9 leptospirosis cases were observed in the Regional Centre for Leptospirosis of Palermo.
The patients were all males (age between 22 and 59 years) and their occupations varied. Laboratory diagnosis is perfomed by the classical microagglutination microscopical (MAT) but this test is very complex and time-consuming.
This study compared the classical MAT with ELISA IgM by using 19 serum samples from 9 patients with confirmed leptospirosis.
We also tested 23 serum samples from blood-donors and 29 serum samples from patients with other infectious diseases.
By the MAT and the PanBio IgM ELISA all sera from patients were found to be positive.
Our results indicate that MAT represents the test with the highest degree of specificity (100%), but ELISA is simpler to perform, considering the favourable degree of sensitivity (100%) and specificity (95.9%).

KEY WORDS: Leptospira spp., Microscopic Agglutination Test, IgM-ELISA

Received November 11, 2003 *Accepted* December 21, 2003

INTRODUCTION

Leptospirosis is a zoonosis with a worldwide distribution very common in most countries.
This acute febrile illness, is caused by microorganisms of the genus *Leptospira*.
At the moment, more than 200 pathogenic *Leptospira* serovars have been identified.
In temperate climates the risk of acquiring the disease is associated with occupational factors or with recreational exposure, through indirect contact with the urine of infected host animals (Mansueto and Trimarchi, 1984), (Ciceroni *et al.*, 1988), (Cacciapuoti *et al.*, 1994). Early diagnosis of leptospirosis is important, since a delayed diagnosis could have a high mortality rate among the patients with the most severe presentations.
According to data of a national epidemiological study performed in the period 1994-1996 (Ciceroni *et al.*, 2000), the number of cases of Leptospirosis in Sicily was lower than that in Northern and central regions of Italy. In fact in the same period, regions like Veneto, Lombardia and Piemonte which comprise 30.9% of the State population, reported 73.4% of the total cases versus 7.2% of the Southern regions (Sicily and Sardinia included).
Diagnosis of human leptospiral infection is based

on either isolation of the *Leptospira* from body-fluids of the patient or demonstration of a rise in specific anti-leptospiral antibodies (Gsell, 1990). Isolation, the gold standard for diagnosis, is not always successful and is difficult to perform, especially in not sufficiently equipped laboratories. In addition, samples for diagnostic purposes (blood, urine, etc.) are obtained in general when microorganisms are not demonstrable peripherically (Ciceroni *et al.*, 1989). Other more recent DNA-based methods, are not routinely applied. Consequently laboratory diagnosis of leptospirosis is based on serological methods. The main methods for this purpose are the microscopic agglutination test (MAT) which is the cornerstone of the serodiagnosis of this illness (Gussenhoven *et al.*, 1997), the Enzyme linked immuno-sorbent assays (ELISAs) (Ferdico *et al.*, 1988), and the indirect fluorescent-antibody tests (Appassakij *et al.*, 1995).

Although the microscopic agglutination test (MAT) is the reference test for diagnosis, this test is time consuming, requires a specialized operator for interpretation and the use of a battery of leptospires belonging to different serovars, and hence the maintenance of stock cultures; in addition, the use of live organisms creates a risk of laboratory-acquired infection.

In alternative (or in addition to MAT) an immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) (Winslow *et al.*, 1997) is often used in routine diagnostic laboratories.

The method bypasses most of the difficulties encountered with MAT. In addition, and interestingly, ELISA may assay a great number of samples and gives a less subjective result than MAT.

In this study we evaluate the clinical and laboratory features of the patients and moreover the serologic diagnosis of Leptospirosis, by comparison of the performance of the classic MAT versus a rapid commercially available ELISA for the detection of leptospiral-specific IgM.

In the other side of medal, this ELISA Kit includes only antigens obtained from some serovars (Canicola, Djiasiman, Kremastos, Nikolaevo, Celledoni, Grippotyphosa, Copenhageni, Szwajizak), not necessarily circulating in all countries. The present paper compares MAT and an ELISA method obtained from the market to evaluate:

a) the applicability also in our setting;
b) the kinetics of antibody response in our patients.

MATERIALS AND METHODS

Patients

Between January 1990 and December 1999, a total of 9 leptospirosis cases were observed in the Regional Centre for leptospirosis of Palermo.

The patients were all males, and their age was between 22 and 59 years.

The case distribution according to sex and age revealed that leptospirosis is a disease predominantly affecting adult males of working age (15-64 years).

The occupation of the patients varied; two were unemployed, 1 was retired, 1 a construction worker, 1 farmer, 1 fisherman, 1 sewage worker, and 1 animal keeper.

Five person's occupations involved contact with animals and/or polluted waters.

The probable source of infection and the mode of exposure were estabilished for 6 of the 9 cases of confirmed Leptospirosis. In fact, the source was contact with animals for 3 cases, and contact and/or ingestion of polluted water for 3 cases. Incriminated water sources of leptospirosis infection were fresh water (lettuce 1 case), and stagnant waters (2 cases).

Sera

19 serum samples were obtained at different stages of disease (from 10 to 30 days from the start of symptoms) from 9 patients with confirmed leptospirosis.

Blood was collected by venipuncture and was allowed to clot. Samples were centrifuged and serum was collected and stored at –20°C until processed.

In addition, we also tested 23 serum samples from blood-donors and 29 from patients with leishmaniasis, brucellosis, toxoplasmosis, cytomegalovirus (CMV)-infection, rickettsiosis, echinococcosis, salmonellosis and HIV-infection.

As controls, validated positive and negative sera obtained from the National Reference Centre for Leptospirosis (Istituto Superiore di Sanità, Rome) were also included.

MAT

All sera were tested using suspensions of 19 live leptospiral strains (serovars are given in parentheses): Mezzano I (*Pomona*), Pavia 1 (*Bataviae*), Moskva V (*Grippo typhosa*), Bianchi 1 (*Icterohaemorragiae*), Ballico (*Australis*), Topino 1 (*Sejroe*), Riccio 37 (*Lora*), Castellon 3 (*Castellonis*), Hardjoprajitno (*Hardjo*), Wijnberg (*Copenhageni*), Akiyami A (*Autumnalis*), Riccio 2 (*Bratislava*), Mus 24 (*Saxkoebing*), Hebdomadis H (*Hebdomadis*), Alarik (*Canicola*), Zanoni (*Zanoni*), Poi (*Poi*), Mitis Jhonson (*Tarassovi*), Sari (*Mini*). Each sample of serum was tested at a 1:100 dilution with the single strain by incubating at 33°C for 90 min. After this time, the degree of agglutination was determined by dark-field microscopy examination at a final magnification of 25 X. The endpoint antibody titre was defined as the highest dilution that agglutinated 50% or more of the leptospires.

The positive sera were titrated with the specific serovar(s) with wich they reacted, using the following dilutions: 1:100, 1:320, 1:1000, 1:3200, 1:10000, 1:32000.

Leptospira infection was diagnosed if a fourfold increase in serum titre was observed indicating a seroconversion, or if the titre was ≥1:320 in one serum sample.

ELISA

We used a commercial Enzyme-Linked Immuno-Sorbent Assay for detection of IgM antibody (PanBio, Brisbane, Queensland, Australia) according to the instructions of the manufacturer. The microwells were coated with the following *leptospira* antigens: Canicola, Djiasiman, Kremastos, Nikolaevo, Celledoni, Grippotyphosa, Copenhageni, Szwajizak.

Before testing, all sera were treated with IgG-removal (Stellar, Medical Systems S.p.A., Genova, Italy) and diluted 1:100 in the diluent provided with the ELISA kit.

Briefly, 100 µl of each dilution were transferred to leptospira antigen–coated microwell strips, including cut-off calibrator and control sera, and incubated for 30 min at 37°C.

After washing six times with wash buffer, bound IgM was detected using 100 µl/well HRP conjugated anti-human IgM (Horseradish peroxidase conjugated sheep anti-human IgM) for 30 min at 37°C and after washing again, 100 µl/well TMB (A mixture of 3, 3', 5, 5'-tetramethylbenzidine and hydrogen peroxide) substrate for 10 min at room temperature.

The reaction was stopped by the addition of 100 µl/well of 1M phosphoric acid per well and read at 450 nm with a microtiter plate reader (Metertech Σ 960, Medical Systems S.p.A., Genova, Italy), with a reference filter at 600 nm.

Positivity was determined by comparison with a reference serum sample (cut-off calibrator).

PanBio units were obtained by calculating the ratio of the cut-off absorbance to the sample absorbance and multiplying by 10.

A positive sample was defined as having >11 PanBio units.

RESULTS

Clinical symptoms and signs are reported in table 1. Except for fever, jaundice and hepato/splenomegaly present in all patients, other data are less present.

The laboratory features identified in the 9 cases were: high blood nitrogen (9), high

TABLE 1 - *Clinical features of 9 patients with Leptospirosis*

Symptoms and signs	*No of patients*
Fever	9
Jaundice	9
Hepato & splenomegaly	9
Exanthema	2
Renal failure	2
Vomiting	2
Abdominal pain	2
Diarrhoea	2
Arthromyalgias	1
Confusion	1
Meningitidis	1
Influenza like symptoms	1

TABLE 2 - *Laboratory features of patients*	
Laboratory features	*No of patients*
Hyperazotemia	9
Hyperbilirubinemia	9
Hyperamylasemia	4
Hyperlipasemia	4
Hypertransaminasemia	4
Hypercreatininaemia	4

bilirubin (9), increased levels of creatinine (4), amylase (4), lipase (4) and transaminases (4) (Table 2).

By the MAT and the PanBio IgM ELISA all sera from patients with leptospirosis were found to be positive (Table 3).

MAT found the largest serum-positivity for the following strains: Bianchi, Wijnberg, Riccio 2, Alarik, Ballico, Hebdomadis, Castellon (minimum titre 1:320, maximum titre 1:32000).

All 29 sera from patients with diseases other than leptospirosis were found negative by the MAT and 2/29 (6.8%) were found positive by the PanBio IgM ELISA (1 rickettsiosis and 1 toxoplasmosis).

TABLE 3 - *Comparison of results by ELISA and MAT for Leptospirosis*

			ELISA		*MAT*	
Group		*TOTAL*	*POS*	*%*	*POS*	*%*
A	Blood donors	24	1	4,1	0	0
B	Other pathologies	29	2*	6.81	0	0
C	Patients	19	19	100	19	100

*1 Rickettsiosis and 1 Toxoplasmosis

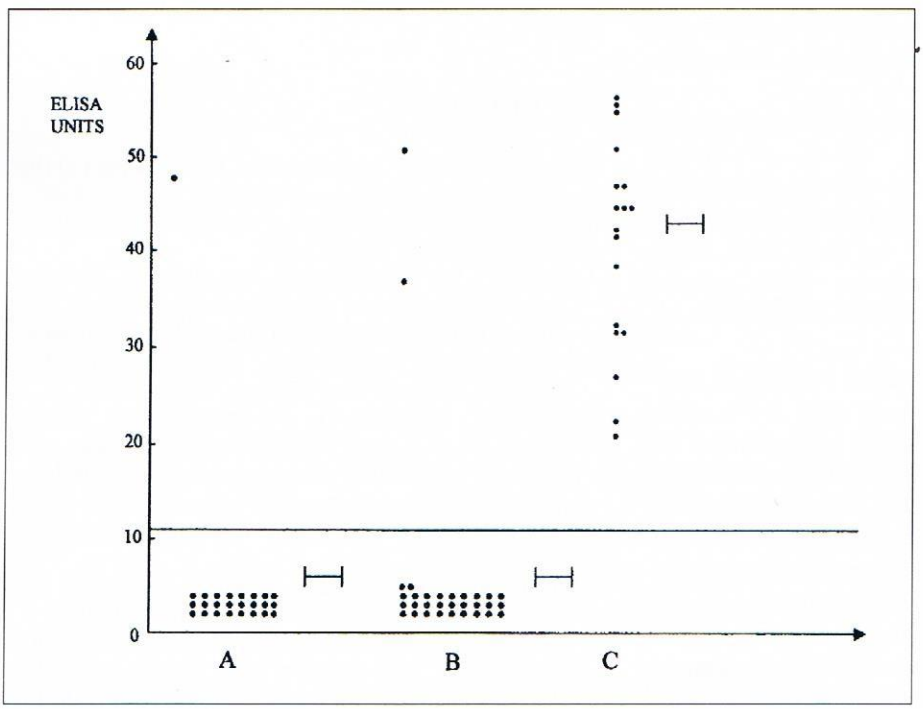

FIGURE 1 - *Results of ELISA test for Leptospirosis Horizontal bars represent arithmetical mean (± SE) for three groups.*
A. Blood donors ($\bar{x}$ = 5.25; SE = 1.84).
B. Other pathologies ($\bar{x}$ = 5.05; SE = 2.02).
C. Patients with Leptospirosis ($\bar{x}$ = 41.07; SE = 2.4).

The 24 serum samples from blood donors were found negative by the MAT and 1/24 (4.1%) was found positive by the PanBio IgM ELISA. Results of ELISA (PanBio units) are reported in Figure 1.

ELISA SPECIFICITY

The sensitivity of ELISA was 100% (19/19) and specificity 93.1% (other pathologies) and 95.8% (blood donors).
The PPV (Positive Predictive Value) was 95%, and the NPV (Negative Predictive Value) 100%

DISCUSSION

The low number of clinically and serologically identified cases of Leptospirosis in this paper confirm the fact that Leptospirosis is a sporadic disease in Sicily, and generally in southern regions of Italy. This sporadicity represents a pitfall because symptoms and signs of the disease are irrelevant for diagnostic purposes especially in non endemic areas (Mansueto and Trimarchi, 1984).
However an adequate surveillance of this pathology is very important especially but not only because the aetiological situation is variable with changing roles of different reservoirs as sources of human infection.
In emergencies (i.e. natural disasters, drought, earthquakes mainly in cases of flood) (Anonymous, 2000) contacts with rat and mouse populations may increase, and on the other hand, the vaccination pressure on dogs and other domestic animals decreases with related increased *leptospira* circulation (Baldelli *et al.*, 2000).
Although performed on a limited number of specimens, our results agree with data from other authors evaluating the same commercial kit (ELISA sensitivity 100%; ELISA specificity for other pathologies 93% and for blood donors 98%) (Winslow *et al.*, 1997) and confirm that MAT represents the test with highest degree of specificity. ELISA is nevertheless more simple and less dangerous to perform and, more importantly, also in our setting presents a favorable degree of specificity and sensitivity, especially when evaluated in association with the clinical symptoms and signs.
We can also conclude that the commercial kit we assessed, although performed with leptospira circulating prevalently in Australia, is a suitable screening test for the detection of IgM leptospira antibodies versus *leptospira strains* circulating in Sicilian territory and probably may be employed in other countries, although MAT remains the only valid laboratory diagnostic method.
In conclusion, our results support the use of this test in Italy and elsenhere although its antigenic composition is mostly constituted by not local strains.
In an attempt to elucidate the comparison between MAT and leptospira IgM currently, a study is being performed in a greter number of patients, including non Sicilians, who have shown a positive MAT for species other than those present in our patients. This study is performed in collaboration with other Regional Referring Centers and also with the National Reference Center (Istituto Superiore della Sanità).

ACKNOWLEDGMENTS
We thank Dr. L. Ciceroni (National Centre for Leptospirosis, Istituto Superiore di Sanità- Rome, Italy)

REFERENCES

ANONYMOUS, (2000). Leptospirosis, India. Report of the investigation of a post-cyclone outbreak in Orissa, November 1999. *Weekly epidemiological record*, **75**, 217-223.

APPASSAKIJ, H., SILPAPOJAKUL, K., WANSIT, R., AND WOODTAYAKORN, J. (1995). Evaluation of the immunofluorescent antibody test for the diagnosis of human Leptospirosis. *American Journal of Tropical Medicine and Hygiene*, **52**, 340-343.

BALDELLI, R., BATTELLI, G., AND POGLAYEN, G. (2000). Zoonosis and other health problems connected with the coexistence of man-dog-cat in normal situations and in emergencies: *Information Circular-WHO Mediterranean Zoonoses Control Centre*, **49**, 2-6.

CACCIAPUOTI, B., CICERONI, L., PINTO, A., APOLLINI, M., RONDINELLA, V., BONOMI, U., BENEDETTI, E., CINCO, M., DESSÌ, S., DETTORI, G., GRILLO, R., FALOMO, R., MANSUETO, S., MICELI, D., MARCUCCIO, L., MARCUCCIO, C., PIZZOCARO, P., SCHIVO, M.L.,

VARALDO, E., LUPIDI, R., IOLI, A., MARZOLINI, A., AND ROSMINI, F. (1994). Survey on the prevalence of leptospira infections in the Italian population. *European Journal of* Epidemiology, **10**,173-180.

CICERONI, L., PINTO, A., AND CACCIAPUOTI, B. (1988). Recent trends in human leptospirosis in Italy. *European Journal of Epidemiology*, **4**, 49-54.

CICERONI, L., GUARDÌ, M., LIBRIZZI, R., FERDICO, V., LA MENSA, G.L., RUSSO, A., AND PINTO A. (1989). Isolamento di Leptospire nel palermitano. *Rivista di Parassitologia*, **3,** 53-56.

CICERONI, L., STEPAN, E., PINTO, A., PIZZOCARO, P., DETTORI, G., FRANZIN, L., LUPIDI, R., MANSUETO, S., MANARA, A., IOLI, A., MARCUCCIO, L., GRILLO, R., CIARROCCHI, S., AND CINCO, M. (2000). Epidemiological trend of human leptospirosis in Italy between 1994 and 1996. *European Journal of Epidemiology*, **16**, 79-86.

FERDICO, V., GUARDÌ, M., AND SALVAGGIO, G. (1988). Ulteriori osservazioni su un test ELISA (IgG e IgM) per Leptospirosi con un antigene del commercio. *Il Patologo Clinico*, **10**, 12-16.

GSELL, O. (1990). The changing epidemiology of leptospirosis in Europe. A report on the 6th meeting of European Leptospira workers, Brno, Czechoslovakia, September 1988. *Zentralbl Bakteriology*, **273**, 412-427.

GUSSENHOVEN, G.C., VAN DER HOORN, M.A.W.G., GORIS, M.G.A., TERPSTRA, W.J., HARTSKEERL, R.A., MOL, B.W., VAN INGEN, C.W., AND SMITS, H.L. (1997). Lepto Dipstick, a Dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in Human sera. *Journal of Clinical Microbiology*, **35**, 92-97.

MANSUETO, S., AND TRIMARCHI, I. (1984). Sintesi della casistica di Leptospirosi in Sicilia. *Acta Mediterranea di Patologia Infettiva e Tropicale*, **3**, 281-283.

WINSLOW, W.E., MERRY, D.J., PIRC, M.L., AND DEVINE, P.L. (1997). Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human Leptospiral infection. *Journal of Clinical Microbiology*, **35**, 1938-1942.