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Commentaries

Leptospira Interrogans Sensu Lato, Etiological Agent of a Zoonotic Disease with Impact on Public Health in Cuba

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Abstract

This paper summarized some interesting aspects related with the microbiological investigations on human leptospirosis carried out in Cuba from 1959 to 2017. The role of the Cuban national reference laboratory on Spirochetes and Brucella in the microbiological surveillance, the confirmation of epidemic outbreaks and in clinical trials related on reactogenicity, immunogenicity, efficacy and effectiveness of the Cuban anti-leptospirosis vaxSPIRAL® vaccine is also assessed. All these results represent contributions to support the National Program of Prevention and Control of the disease in Cuba.

COMMENTARIES

Human leptospirosis in Cuba was poorly described prior to 1959. In the period 1970-1980, a number of nationwide outbreaks occurred, associated with agricultural work in lowland areas infested by rodents, as well as in rivers, dams, canals and water reservoirs contaminated with livestock residues. In 1972, the first World Health Organization recommendation was received, its aim was to implement microbiological diagnosis, with the objective of establishing confirmatory techniques for leptospirosis. Four years later (1978), serological diagnosis by the Indirect Hemagglutination Test (HAT) was made available, in all the provinces of the country. In 1981, the Program for Prevention and Control of Leptospirosis was published and in 1983 anti-leptospiral immunization was initiated in the main risk groups with a Russian vaccine containing serovars from the Icterohaemorrhagiae, Pomona, Grippotyphosa and Hebdomadis serogroups [1]. One of the first descriptive studies was carried out with data available from the National Statistics Office of the Ministry of Public Health. It investigated 397 deaths of leptospirosis, from 1987 to 1993, and reported a sharp increase in morbidity rate, reaching a value of 1.03 / 100.000 inhabitants, the highest in over 50 years. The most affected population group was retirees, followed by production workers and agricultural workers [2].

A national action plan was therefore established in 1994, to reduce the morbidity caused by leptospirosis, with a national incidence rate of 25.6 / 100.000 [3]. In this context, the first Cuban vaccine against human leptospirosis (vaxSPIRAL®), was developed by the Vaccine Research and Production Center of the Finlay Institute. During the vaxSPIRAL® preclinical trial the formulation of vaccine adsorbed on aluminum hydroxide was shown in the hamster model to be safe, immunogenic and protective [4]. The effectiveness of vax-SPIRAL® was 97%. The clinical trial of phase IV was conducted in Holguín, using a prospective, quasi-experimental cohort study in groups at risk of leptospirosis, where 101.137 subjects were immunized with two doses of 0.5 mL, 6 weeks apart (cohort of vaccinates), aged between 15 and 65 and with permanent or temporary risk of contracting the disease, 16 881 participants were not immunized (unvaccinated cohort) [5].

The clinical trial of reactogenicity (Phase I) and immunogenicity (Phase II) of vax-SPIRAL® was undertaken with healthy volunteer groups from Havana. The Russian vaccine was used as a control. There were no serious adverse reactions and low titers of agglutinating antibodies against leptospires were found in the adult volunteers [6]. The vax-SPIRAL® efficacy was 78.1% (95% CI: 59.2 to 88.3) and the relative risk of contracting leptospirosis infection between vaccinated and unvaccinated individuals was 0.22 (95% CI: 0.12 to 0.41). This study of phase III was performed in a controlled, randomized, double-masked manner in Villa Clara using as a control the Heberbiovac-HB recombinant hepatitis B vaccine (Heber Biotec, Cuba) [7].

The sera from individuals who were vaccinated (vaxSPIRAL®) or received the placebo, from phases I and II were studied by the ELISA [8]. Results showed (phase I) that out of 38 vaccinees, 11 seroconverted (29%) by MAT and 12 (32%) by ELISA. Out of 33 subjects receiving the placebo, two (6%) and three (9%) seroconverted, respectively. In phase II, out of 68 persons

vaccinated with a dose of 0.25 mL and 65 with a dose of 0.50 mL, the seroconversion rate was 21 (31%) and 16 (25%) by ELISA, and by MAT 9 (13%) and 7 (11%) respectively. In most reactive individuals, antibody levels were found by MAT against at least one of the vaccine strains. However, the level of agglutinating antibodies detected with respect to the vaccine-induced protection in sera could not be correlated in this investigation. Therefore, it is recommended to apply the passive immunization test to measure the level of protection of the vaccine [9]. Other research demonstrated a high seroprevalence of antibodies (by ELISA-IgG), against the vaccine serovars before the start of the study, and an increase to twice the initial value of the antibody response at 21 days after the second dose of the vaccine, in 45% of individuals, with markedly significant differences (p= 0.000000). Antibody levels declined after one year. However, 23.1% of subjects still showed levels that were double those before vaccination. Based on these results, it was decided to administer the vaccine to the main risk groups [10].

An outbreak of human leptospirosis occurred in 1997 (Villa Clara City).This situation was associated with predisposing epidemiological factors in the affected population. The infecting serogroups found were Ballum, Pomona, Canicola and Icterohaemorrhagiae. Sera from seven suspected cases and four severe patients were confirmed as leptospirosis by HAT and MAT. Two isolates were typed as Ballum. Two urine and one blood culture were positive by PCR [11].

From 1998 to the present, strict surveillance of human leptospirosis has been maintained. The national incidence is an average of 980 patients / year (8.8 / 100.000) with around 25 deaths was reported [12]. However, the occurrence (in 1999), of the hurricanes George and Mitch in some Cuban provinces, accompanied by significant rainfall and flooding resulted in new outbreaks, particularly in Pinar del Río, Las Tunas and Havana. The main cause of infection was contact with rodents and the failure to take protective precautions [3]. Another leptospirosis epidemic event occurred in Havana (2001), associated with the city's lack of adequate sanitation. A descriptive analytical study was carried out on 240 suspected patients from IPK' hospital, of which 33 were confirmed microbiologically. By typing, the Ballum serogroup was predominant, whereas serologically (MAT) Canicola, Sejroe and Icterohaemorrhagiae were the most frequent ones. The predominant clinical form was anicteric (73%), and the principal symptomatology observed were fever (88%), headache (73%), and myalgia (67%). Bilirubin and creatine phosphokinase (CPK) levels were elevated in 83.3% and 67% of cases, respectively values. Almost 73% of the confirmed were not related to occupations at risk [13].

After that outbreak there was a clear reduction in the leptospirosis morbidity rate (2.7 / 100.000) and again, between 2005-2006, the incidence rates increased (6 and 7.4 /100.000 respectively). The use of a warning system applied in the provinces of Guantánamo and Santiago de Cuba, detected a new epidemic in 2005, coincident with the significant increase in rainfall and floods caused by Hurricane Wilma. From 885 suspected cases, 61 (6.9%), were confirmed microbiologically [14]. *Leptospira* isolates were typed as Canicola, Ballum, Icterohaemorrhagiae and Pomona serovars [15].

From a study carried out in two municipalities of Havana city, a slight increase in lethality due to leptopirosis was observed, with case fatality reaching more than 46%. Concomitantly, the morbidity rate reached 0.54 / 10^5 mortality of 0.28 / 10^5 [14] [15].

Several laboratory techniques had been evaluated for the diagnosis of the disease since the 1980's. One of the first studies was the validation of the HAT using sera from patients and personnel with occupational biological risk [16]. In the same year, leptospires were isolated from 32.7% of the waters, with three strains *L. biflexa* and three such as *L. interrogans*, serogroups Australis (2) and Icterohaemorrhagiae (1) [17]. The slide agglutination technique was used in 1990 and found 55% of seropositive cases [18]. In 1991, leptospiral antigens were produced for use in the counterimmunolectrophoresis technique, achieving a sensitivity of 82% and a specificity of 100% [19]. In the same year, antigens of *L. interrogans* were used in immunofluorescence (IFI) achieving a slightly greater sensitivity of 86.5% [20]. Another study from 1992 reported the use of three variants of the HAT, and it was observed that the variant with formalinized human blood of the O negative group had a sensitivity of 84%, a specificity of 98% with a good reproducibility and repeatability [21]. In 1993, a study of three variants of IFI reported a sensitivity of 80% [22]. However, a study from august 1993 to January 1994, on 500 presumptive patients with leptospirosis found only 28% serological positivity by HAT [23]. The same year the use of the ELISA system for the detection of total antibodies against leptospires was reported for the first time in Cuba, with a 65% concordance with MAT [8]. However, using a higher number of sera in 1995, a 100% agreement with MAT was reported [24]. The Cuban ELISA was applied (1996) to different sera groups and showed a concordance of 89% with HAT and 65% with MAT [25]. After that, a technical variant of MAT, using 1:10 as first dilution of the sera was developed. The sensitivity of MAT-V was of 78% and the specificity of 89%. The Ballum serogroup was prevalent in positive sera by MAT-V, supporting the use of this variant to detect patients with low levels against others leptospire serovars [26]. Shortly after (2004), variants of slide agglutination systems with specific leptospire serovars / latex particles were studied for the detection of specific antibodies in human and animal sera. The antigenic concentrations were adjusted between 1.4 and 1.5 $X10^9$ cells / mL. The Cuban latex-pool presented acceptable values of sensitivity (94%) and specificity (90%), and was reproducible and stable for 6 months [27]. The evaluation of the immunochromatographic lateral flow system showed a concordance of 98% with MAT [28,29]. A new home made immunochromatographic system was developed in 2015, using an antigen mixture of the five serogroups of leptospires predominant in the country. It showed a sensitivity of 96%, a specificity of 97.1% and a concordance with MAT of 96.7% [30]. More recently a commercial system of SD Leptospira ELISA-IgM (BIO-LINE Standard Diagnostics, INC) was applied in 2011, which showed more than 90% agreement with MAT. The system enhanced the rapid diagnosis of human leptospirosis and active laboratory surveillance in all Cuban provinces [31].

The first molecular diagnoses for early detection of DNA from *Leptospira* spp in blood cultures from suspect patients was

Table 1: Main results of epidemiological data and laboratory surveillance of human leptospirosis in Cuba (1959-2017).

Epidemiological data	Laboratory data
1959-1971: occurrence of nationwide leptospirosis outbreaks mainly associated with agricultural work [1].	
1972: laboratory recommendations from WHO expert team on leptospirosis visiting Cuba [1].	**1978**: implementation of HAT in all Cuban provinces [1].
1981: publication of the Cuban Program for Prevention and Control of Leptospirosis [1].	
1983-1992: first use of Russian vaccine in Cuban risky groups [1].	**1986**: validation of HAT technique [16]. Isolation of pathogenic and non-pathogenic Cuban leptospires [17].
	1990: use of slide agglutination technique [18].
	1991: evaluation of CIE [19] and IFI techniques [20].
	1992: use of the formalinized human blood of the O negative group in HAT [21].
1993: a sharp increase in morbidity rate (1.03 / 100.000). Retirees, production workers and agricultural workers were most affected [2].	
1993-1994: another increase in the incidence rate of the disease (25.6 / 100.000), with a sustained lethality of 1.8%. **1994**: Establishing of a national action plan to reduce the morbidity. Began studies to develop vaxSPIRAL® Cuban vaccine [3].	**1994:** first use of ELISA system for diagnosis of leptospirosis [8], and its application to Cuban risk groups [24] **(1995)**.
1997: outbreak in Villa Clara City associated with predisposing epidemiological factors [11].	**1997**: identification of the serogroups Ballum, Pomona, Canicola and Icterohaemorrhagiae [11].
1999: two outbreaks associated with hurricanes Georges and Mitch [3].	
	2000: evaluation of a MAT-V variant technique and predominance of Ballum serogroup [26]. Clinical trial of effectiveness of vax-SPIRAL® vaccine [5].
2001: outbreak of leptospirosis in Havana [13].	**2001**: identification of the serogroups Ballum, Canicola, Sejroe and Icterohaemorrhagiae [13].
	2002: evaluation of the immunochromatographic lateral flow system [28,29]. Identification of the serogroups Ballum, Pomona, Canicola, Pyrogenes, Autumnalis and Bataviae [40]. Serologic and genetic study of leptospires [41]. Clinical trial of reactogenicity /immunogenicity of vax-SPIRAL® vaccine [6].
	2004: preclinical trial of vaxSPIRAL® vaccine [4]. Evaluation of a Cuban latex-pool agglutination test [27]. Clinical trial of efficacy of vax-SPIRAL® vaccine [7].
2005-2006: a warning system detected an outbreak in Guantanamo [14].	**2005-2006**: identification of serovars Canicola Canicola, Ballum Ballum, Icterohaemorrhagiae Icterohaemorrhagiae and Pomona Pomona [14]. First investigation of Cuban leptospire isolates to several antibiotics by MIC [42]. Seroprevalence study of vaccinated individuals with vax-SPIRAL® vaccine [10].
2006: it was founded in Havana a slight increase in lethality (more than 46%) to leptopirosis [15].	**2006-2008**: identification of the serogroups Pomona, Canicola, Icterohamorrhagiae, Ballum , Hebdomadis and Lousiana [43].
	2007: first use of PCR for early diagnosis of the disease in Cuba [32]. Identification of the serovars Pomona Pomona, Pomona Mozdok and Canicola Canicola [44].
	2008: application of PCR in postmortem diagnosis of leptospirosis [33].
	2012: study of leptospiral OMPs as potential vaccine candidate [37].
	2013: application of SD Leptospira ELISA-IgM in Cuba [31].
	2014: PCR optimization with novel primers [34, 35]. Use of Chelex-100 method for DNA extraction in clinical samples [36].
	2015: evaluation of a Cuban immunochromatographic system for diagnosis [30]. Detection of protease and nuclease activity in Cuban isolates [38].
	2016: identification of the serovars Ballum Guangdong, Ballum Arborea, Ballum Ballum, Pomona Pomona, Pomona Mozdok, Pomona Proechimis, Canicola Canicola, Icterohamorrhagiae Copenhageni and Icterohamorrhagiae Icterohamorrhagiae in different provinces [45].

published in Cuba in 2007, showing high diagnostic specificity [32]. One year later (2008), this system was used for the postmortem diagnosis of leptospirosis using 171 fresh tissue samples from organs of 50 deceased suspects and 32% were confirmed. In this paper, the lung showed a positivity of the 25% (12 / 48); the liver of 25.6% (11 / 43); the kidney of 22.5% (9 / 40); the brain of 6.3% (1 / 16); the heart 7.1% (1 / 14); the spleen of 12.5% (1 / 8) and the muscle of 50% (1 / 2) [33]. After that, the PCR was optimized by adjusting the hybridization temperature and the concentration of primers. Standardized PCR with novel primers was specific and more sensitive than the reference PCR [34,35]. In addition, a comparison of DNA extraction methods for pathogenic leptospires (QIAmp DNA mini kit (QIAGEN, Germany), CLART HPV kit (GENOMICA, Spain) and Chelex-100), demonstrated that the last one was the most efficient for DNA extraction in clinical samples for use in PCR [36].

A basic leptospirosis research developed has involved the study of bacterial outer membrane proteins (OMP) as potential vaccine candidates. The Triton X-100 method was used for the extraction of these proteins from the Castellon 3 reference strain of the Ballum serogroup, Serovar Castellonis. Different concentrations were tested and the rates and times of the extraction were modified. The occurrence of cell lysis through PCR and zymography, as well as the presence of lipopolysaccharides by proteinase K assay, were explored in the protein extracts [37]. In 2015, an efficient method was described for the detection of protease and nuclease activity in leptospiral isolates. Extracts of ten strains of leptospires (nine pathogenic and one non-pathogenic) were evaluated and protease activity was observed with different behavior between the pathogenic strains and the non-pathogenic strain [38].

During this time frame, several studies have attempted to characterize the Cuban serovars of leptospires. One of the first reports was carried out in 1996, typing leptospiral strains from Holguin, where the Ballum, Canicola and Pomona serogroups predominated [39]. Later (2002), 204 strains of *L. interrogans* pathogenic complex, from different regions of the country were studied, showing that serogroups Ballum, Pomona, Canicola, Pyrogenes, Autumnalis and Bataviae were the most prevalent. These findings identified mice, pigs and dogs as the main reservoirs [40]. At the same time, 18 strains of *Leptospira*, isolated from three provinces of Cuba, were studied by serological and genetic methods [41].

A method to determine the minimum inhibitory concentration of a range of antibiotics against leptospires was developed in 2005. Reference strains of the pathogenic complex *L. interrogans sensu lato* and *L. biflexa sensu lato* were tested against penicillin, ciprofloxacin, chloramphenicol, rifampicin and tetracycline. The results showed lowest values for ciprofloxacin, rifampicin and tetracycline and the highest values with chloramphenicol and penicillin [42].

A new report (2006-2008) demonstrated as the predominant serogroups, Pomona (11) 42.3%, Canicola (6) 23.1%, Icterohamorrhagiae (5) 19.2 %, Ballum (2) 7.7%, Hebdomadis and Lousiana (1) 3.8% [43]. Sixteen of the isolates were then typed with monoclonal antibodies as Pomona Pomona, Pomona Mozdok and Canicola Canicola [44]. The most recent study in Cuba embraced a total of 79 isolates (from Las Tunas and Holguín provinces) where the predominant serovars were Ballum Guangdong, Ballum Arborea, Ballum Ballum, Pomona Pomona, Pomona Mozdok, Pomona Proechimis, Canicola Canicola, Icterohamorrhagiae Copenhageni and Icterohamorrhagiae Icterohamorrhagiae. Eleven untyped isolates were confirmed as pathogens by PCR based on the pathogen-specific *lipL*32 gene [45].

In summary, the main results of epidemiological data and laboratory surveillance of human leptospirosis in Cuba appeared in table 1. All them support the national program for prevention and control of leptospirosis and to a better understanding of the etiological agent and its circulation in the country.

Finally, since 2001 and every two years, the reference laboratory of Spirochetes and *Brucella* have made several scientific meetings [46-49], aimed at the theoretical - practical training of human resources on leptospirosis. More than 400 colleagues and experts from America, Europe, Asia and Australia have participated. Also consultancies have been received by ILS / WHO 'experts in the leptospirosis field.

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