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Short Communication

Biochemical, serological and genetic identification of Leptospira isolated from Cuban patients (2008-2009)

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Keywords

- *Leptospira*
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Abstract

Introduction: Identification of pathogenic *Leptospira* is important for the formulation of epidemiological studies, including the design of new vaccines, and the validation of rapid diagnostic tools. In Cuba there are few studies of typing leptospireserovars circulating in different geographical region through the years.

Objective: To contribute to the knowledge of the *leptospira* serovarscirculating in Cuba using biochemical, serological and molecular tests.

Materials and methods: Seventeen autochthonous isolates of leptospirosis cases from two cuban'sprovinces (2008-2009) were studied using for the identification, the biochemical tests of 8-azoguanine resistance, the low temperature growthing (13°C) and the sensitivity tothe 1% sodium bicarbonate. MAT technique using polyclonal and monoclonal sera and PCR were also used.

Results: Ten (10/17) isolates were typed by MAT with pyloclonal sera as Pomona (4), Canicola (4), andIcterohaemorrhagiae (2) serogroups. Belong these serogroups, Pomona (1),Tropica (1), Proechimys (2), Canicola (4), and Icterohaemorrhagiae (2) serovars were identified using MAT with mAbs. All 10 autochthonous isolates amplified DNA's fragments by PCR test. Seven (7/17) isolates were not typed by MAT, however all them were identifiedbiochemically and molecularly as pathogenic strains.

Conclusions: The distribution of leptospire serovars from 2008 to 2009 in Cuba, had been similar behavior with respect to previous periods, which suggests maintaining laboratory surveillance of leptospirosis.

INTRODUCTION

Leptospirosis is one of the most wide spread zoonotic diseases in the world. The epidemiology of leptospirosis is complex and dynamic and there is a wide variety of (a) specific clinical manifestations with a broad differential diagnosis that makes diagnosis difficult [1].

The initial identification of *Leptospira* (bacteria causing leptospirosis) is morphological, by dark field microscopy. Detection of *Leptospira* by culture constitutes the definitive diagnosis; however, it is hampered by slow growth rates of some *Leptospira* strains and the long incubation periods before an isolate is established in culture.Definitive identification of the isolates requires the use of serological and molecular techniques. Based on serological criteria, strains of *Leptospira* are differentiated into serovars,which represent the basic taxon. Serovars that are antigenicallyrelated, are placed intoserogroups. Serogroups do not have an official taxonomic status, but are of clinical andepidemiological importance. Currently more than 200 pathogenic serovars have beenarranged in 26 serogroups. The genotypical classification system is based on DNA homology.In this scheme, *Leptospira* are placed into 20 *Leptospira*species of a pathogenic, saprophyticand intermediate nature. There is a poor correlation between the serological andgenotypic classification systems [2].

Pending the development of a whole classification system based on the new molecular typing methods, the InternationalCommittee on Systematic Bacteriology Subcommittee on the Taxonomy of *Leptospira* recommended that

new field isolates should still be typed by the recognized standard method, the cross-agglutinin absorption test (CAAT), and given serovar status; however, this technique, time-consuming and is very difficult, for that other valid methods can be used for identification. The recommended methods include analysis with monoclonal antibodies (mAbs) or factor sera [3].

Cuba has a National Program for the Prevention and Control of leptospirosis since 1981, which brings together multiple spheres of social, political and economic life. During the period 1988-1990, an average of 778 annual cases were reported. In 1991 most provinces increased their morbidity and mortality rates and in 1994 the highest epidemic peak of the disease occurred, with an incidence rate of 25.6 per 100 000 inhabitants and a lethality rate of 1.8%. In 1995, 2169 cases were reported, for a rate of 19.5 per 100 000 inhabitants, with 60 deaths, and from 1996 to 1997 where 1085 cases were reported (morbidity rate of 9.8) and 52 deceased. In 1998, 980 cases were reported (with a rate of 8.8 and with 25 deaths). Since 1997, anti *leptospirosis* immunization programs have been applied, mainly to groups of risks. Since 1998 till now, a strict surveillance of leptospirosis has been maintained throughout the country. In all this time the morbidity and mortality indicators is maintain around 500 cases annually [4].

The National Reference Laboratory on *Leptospira* of IPK implements the use of new laboratory tools to type of leptospire serovars circulatingin Cuba in order to contribute to the knowledge of the etiological agent in our environment. Therefore, for this researchthe aims are to implement a laboratory using biochemical, serological and PCR tests for typing some Cuban leptospireserovars.

MATERIAL AND METHODS

Leptospira strains

seventeen autochthonous isolates of leptospirosis cases from two cuban's provinces (Las Tunas and Holguín) were received at the National Reference Laboratory on *Leptospira* of the IPK, from 2008 to 2009.They are the strains345LT; 265LT; H1108; H-27; H792; 225LT; H487; 489LT; H808; H807; H-1356; H-473; 91-LT; 121-LT; 80-LT; H-1065; and 234-LT. Also, was used areference strain: *L. interrogans* Pomona Pomona Pomona, as control.

***Culture*:** Culturing was performed in liquid Ellinghausen and McCullough modified Johnsonand Harris (EMJH) and semi-solid Fletcher culture medium (conservation).Incubation was at 30°C and cultures were inspected by dark field microscopy for growth of *Leptospira* at regular intervals. Isolates were sub-cultured and maintained in EMJH mediumsupplemented with 5 fluorouracil (200µg/ml) as a selective inhibitor for contaminating micro-organisms [5].

Phenotypical tests

Resistance to 8-azoguanine test, the growth at low temperature (13°C) and the sensitivity to 1% sodium bicarbonate, were made according to ILS-WHO protocols for *Leptospirosis* [5]. Three replicates were made, for each autochthonous isolates and also to the three reference strains were made.

Serological typing

Each autochthonous isolates was typed by *MAT* with group sera and monoclonal antibodies. Rabbit anti-*Leptospira*sera were raised following standard procedures. To identify the isolates up to serogroup level, MAT was performed following standard procedure using a panel of 11 anti-*Leptospira* rabbit reference sera [5].

Polyclonal rabbit anti-*Leptospira* sera

Polyclonal rabbit anti-*Leptospira*sera employed were from Ballum;Bataviae;Canicola;Cynopteri; Grippotyphosa; Hebdomadis; Icterohamorrhagiae; Pomona; Sejroe;Tarassovi and Semarangaserogroups.

Monoclonal antibodies (mAbs)

Isolates were further typed at the serovar level by performing MAT with panels of monoclonal antibodies (mAbs) to Pomona (F46C1-5, F46C4-3), Mozdok (F48C1-4, F48C6-5), Icterohaemorrhagiae RGA (F70C24, F70C14-10, F70C7-11, F89C12, F52C1-4, F12C3-11), Canicola (F152C7-4, F152C18-4, F152C11-3), Hardjoprajitno (F16C28-3, F16C140-4, F16H327-3, F50C3-4), and Ballum (F74C1-7, F74C4-4, F74C7-4). Serial dilution of antibodies were made in phosphate buffered saline (PBS) pH 7.2 and 50 µl aliquots placed in each well of a microtitre plates to which was added an equal volume of a 7 to 10 days old culture of live *Leptospira* in EMJH medium. After 2 - 4 h of incubation at 37ºC, the agglutination titre was determined by dark field microscopy [5].

PCR-LipL32

Iisolation of chromosomal DNA of all autochthonous isolates was studied by PCR based on the pathogen-specific *lip L*32 gene as previously described [6]. The primers LipL 32-270F and LipL 32-692R, which recognize a conserved region of the gene encoding the outer membrane lipoprotein LipL 32, present only in all pathogenic leptospire strains, were used [6].

RESULTS AND DISCUSSION

None of the isolates grew inEMJH medium with 8-azoguanine, or with 1% sodium bicarbonate, orEMJH medium incubated at 13°C temperature. This result are confirming theirs pathogenicity. All observation by dark field microscopy, demonstrated first, that the control reference strain Patoc I of Semarangaserogroup, grew satisfactorily in these conditions, and second one, that the replicates of the pathogenic control strains Pomona Pomona and Canicola Canicola, were also completely inhibited in presence of with 8-azoguanine, 1% sodium bicarbonate, and at 13°C temperature (after the fourth day).

Pathogenic leptospire strainsincorporatethe pyrimidine's and do not incorporate purines to their DNA, for that, they are inhibited in presence of 8-azoguanine (2-amino-6-oxy-8-aza-purine). Conversely, the saprophytic (non-pathogenic) leptospire strains, grow satisfactorily in presence of the 8-azoguanine [7].

The growth in the presence of the 8-azoguanine, has much

practical value when people trying to differentiate *leptospira* strains isolated from humans, animals, waters or soils. In this work, we were able to differentiate pathogenic strains isolated from humans. Not was possible to use other strains isolated from animals, waters or soils [7].

The EMJH media with sodium bicarbonate (1%) generates a reducing environment for leptospire. The NaHCO3 decreases the oxygen tension, which affects the cellular growth of this bacterium. Pathogenic *Leptospira* are aerobic microorganisms strictly, while saprophytes can survive better in a certain microaerophilic atmosphere. The EMJH media with sodium bicarbonate (1%) actas a toxic growth inhibitor for pathogenic leptospire strains. Non-pathogenic leptospire canmultiply satisfactorily in vitroin EMJH media with sodium bicarbonate (1%) [7]. In this investigation, none of the 17 autochthonous isolates, neither the control reference strains (Pomona Pomona and Canicola Canicola), grew in the media EMJH with 1% sodium bicarbonate.

Growth at 13°C, is a simple laboratory test, which can differentiates pathogenicand non-pathogenic strains. The pathogenic *Leptospira* need an optimal temperature for their growth (28 to 30°C). So they generally persist in humans where the body temperature is around this value. Saprophytic *Leptospira* can grow in freshwater pools, dams, rivers, at temperatures well below 28°C. For that, they are rarely isolated from warm-blooded [7]. Taking into account the results of this paper, none of the 17 autochthonous isolates are growing at this temperature (13°C).

By MAT with polyclonal sera were typed in sero group, ten strains for 58.82% (10/17). Only the 41.17% (7/17) were untyped. Authors describe the lost of the phenotypic characteristics in leptospire strains when is performing successive subcultures. The culture medium influences in the surface antigens of *Leptospira*, causing changes in their structure and biological activity. Only, the syrian golden hamster passes, recover virulence and pathogenicity in strains of *Leptospira* [7].

Pomona (4/10), Canicola (4/10), and Icterohamorrhagiae (2/10) serogrupswere the predominant using MAT with polyclonal sera. The serogroups found in this study coincide with others reported in Cuba since 1996 [8].

All the isolates belongCanicolaserogroupwere identified as Canicolaserovar, using the monoclonal F152C11-3, recommended to identify always this serovar. Table 1 shows the antibody titers of each isolates in front of the monoclonal antibody used toidentifyCanicolaserovar.

All the isolates belongIcterohaemorrhagiaeserogroupwere identified asIcterohaemorrhagiaeserovar,using the monoclonal antibodies F70C14-7 and F70C24-16. Copenhageni and Icterohaemorrhagiae serovars are easily differentiated using the monoclonal F70C14-7, because for Icterohaemorrhagiae serovar the titer found are higher (≥40 960), while Copenhagenithe titer are lows (around 80). This phenomenon is the reverse with the monoclonal F70C24-16, which recognizes the serovarCopenhageni in high titer (≥ 140 960), while Icterohaemorrhagiae the titer are lows (around 160). Table 2 show the antibody titers of the isolates against the monoclonal antibodies used to Icterohaemorrhagiae serovar.

Was typed as Tropicaserovarthe H-487 isolate,which show a titer of 160 in front of the F43 C9-7and of the 80 for F43 C9-5monoclonal antibodies, which bothcombines to identify thisserovar.

As Proechimysserovarwas recognized the 489-LT isolate (with high titer 40 960 in front of F48C2-3, F46C10-1 and F61C7-1monoclonal antibodies). Not being so in front of the F46C4-5, F46C1-4, F46C14-4 and F46C1-1monoclonal antibodies.Also, H-808 isolate was recognized as Proechimysserovar. This strain show higher titers from 1 280 to 20 480, for the F61C7-1, F46C10-1, F46C4-5, F46C1-4, and F43C9-7, and low titers from 80 to 640 in front of the F48C1-4, F46C14-4 and F43C9-5monoclonal antibodies.

With Pomona serovarpattern was recognized the H-807 isolate, with a titer of 640 in front of the F43C9-7monoclonal antibody. This isolate was negative in front of the F48C1-1, F46C4-5, F43C9-5, F46C1-4, F48C2-3, F46C10-1, F61C7-1, F46C14-4 and F46C1monoclonal antibodies.

Pomona and Mozdokserovars, have been frequently found in Cuba between 1995 - 1996. Both serovars are distinguishable with F43C9mAb (showing titre of 160). This monoclonal does not recognize the Mozdokserovar [7,8].

Seven isolates (7/17) were not typed by MAT using polyclonal and mAbs. Previously Rodríguez Y et al., reporter as Pomona sero group the 121-LT and H-1065 isolates [9]. The reason of this contradiction could be the existence of genetic mutations in these strains, after several subcultures in EMJH medium.

However, these sevenisolates were positive by PCR, using primers that recognize the conserved region of the gene that codes for lipoprotein of outer membrane LipL 32. For these isolates were obtained intense bands of DNA with size of the 423 base pairs (bp). See figure 1, lanes: **1**, fragment size marker**; 2**, **strain** H-807 (Pomona serovar); **3,strain** 265-LT(Canicolaserovar); **4,strain** H-792 (Icterohaemorrhagiae serovar)**; 5,strain** H-487 (Tropicaserovar); **6,strain** H-207(Canicolaserovar); **7, strain**H-808(Proechymisserovar); **8, strain** H-11.08 (Canicolaserovar); **9, strain** 225-LT(Icterohaemorrhagiaese rovar); **10, strain** 345-LT(Canicolaserovar); **11, strain** 489-LT (Proechimysserovar); **12, reference strain** *L. interrogans* Pomona PomonaPomona; **13, strain** H-1356; **14, strain** H-473; **15, strain** 91-LT; **16, strain** 121-LT; **17, strain** 80-LT; **18, strain** H-1065; **19, strain**234-LT and **20, negative control** (sterile distilled water).From lanes 13 to 19 are the untyped isolates by MAT with polyclonal sera.

The primers used in this study recognize a conserved region of the gene encoding LipL 32 from the outer membrane lipoprotein, presents only in the pathogenic leptospire strains. This protein was cloned from the genome of the strain *Leptospira interrogans* serovar Bratislava. The recombinant proteins are used as antigens for the detection of antibodies specific for *Leptospira* spp. Recent data show that LipL 32 is expressed in the proximal renal tubules of the urinary system of infected animals. It is also known that LipL 32 is the most notable protein within the protein profile and that it is an immunodominant antigen modulator, present throughout the leptospirosis infection [9].

Table 1: Antibody titers of each isolates in front of the monoclonal antibody used to identifyCanicolaserovar.

Isolates	MAT titers MAb: F152C11-3	Serovar
345-LT	5120	Canicola
265-LT	10240	
H-11,08	160	
H-207	2560	

Source: National Reference Leptospire Laboratory Registry – IPK.

Table 2: Antibody titers of the isolates against the monoclonal antibodies used to Icterohaemorrhagiae serovar.

Isolates	MAT titers		Serovar
	MAb: F70C14-7	MAb: F70C24-16	
H-792	10240	320	Icterohaemorrhagiae
225-LT	20480	320	

Source: National Reference Leptospire Laboratory Registry – IPK.

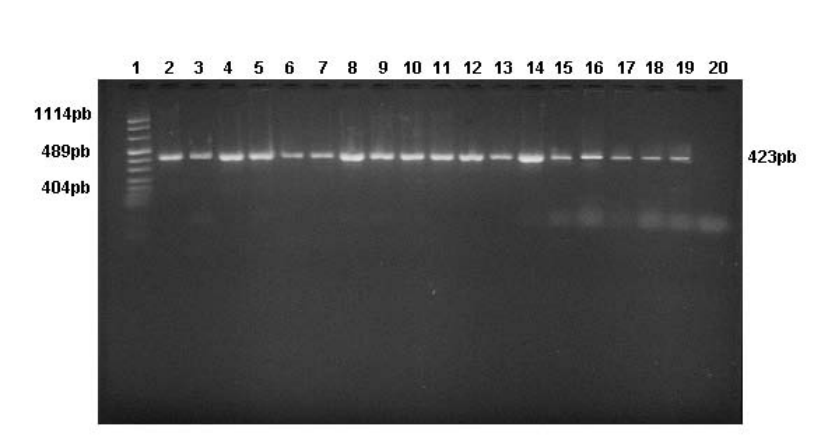

Figure 1 Chromosomal DNA products amplified by PCR method from seventeen autochthonous isolatesand reference strain of serogroupPomona. Lanes: **1**, fragment size marker**; 2**, **strain** H-807; **3,strain** 265-LT; **4,strain** H-792**; 5,strain** H-487;**6,strain** H-207;**7, strain**H-808;**8, strain** H-11.08;**9, strain** 225-LT; **10,strain** 345-LT;**11,strain** 489-LT; **12, reference strain** *L. interrogans* Pomona PomonaPomona; **13, strain** H-1356; **14, strain** H-473; **15, strain** 91-LT; **16, strain** 121-LT; **17, strain** 80-LT; **18, strain** H-1065; **19, strain**234-LT and **20, negative control** (sterile distilled water).

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