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RESEARCH ARTICLE

Decreased rate of *Leishmania* infected *Phlebotomus* captured in Constantine (Algeria) from 2011 to 2013.

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Abstract

Leishmaniasis has been responsible for major public health concern in Algeria from decades. The incidence has previously reached 5000 cases per year, leading to a major medical and economic burden. However, since 2012, health services of Constantine area reported a trend to regression of the leishmaniasis cases number, based on unpublished observations from local health workers. This hypothesis is strengthened by the decrease of the glucantime quantity used to treat patients in different services of Constantine's University Hospital from 2010 to 2013.The objective of this work was to highlight the regression of the disease transmission in Constantine's area, using real time PCR for molecular detection of *Leishmania* parasites from the *Phlebotomus* vector. The sampling sandflies campaigns were conducted from 2011 to 2013 in some localities of Constantine area, leading to capture of 1498 and 4360 females and males *Phlebotomus* respectively. Our results showed a very low number of infected vectors among the population captured. While leishmaniasis transmission is complex and multifactorial, these data provide a first limited evidence of possible success of long term fighting against this disease in the Constantine area.

1. Introduction

Leishmaniasis are classified as neglected infectious diseases, while they cause millions of cases of visceral and cutaneous infections among the world, leading to a high social and medical burden in endemic countries. These parasitic infections, common to human and various animals, are transmitted by the sting of the female sandfly (Dedet, 2009). *Leishmania* parasite induced a large variety of clinical manifestations ranging from cutaneous forms wich resolve spontaneously, to the visceral forms which turn out fatal in the absence of treatment. The annual incidence of leishmaniasis clinical forms was estimated at 1.5 to 2 million new cases per year (Desjeux, 1996). In Algeria, this zoonosis represents 35% of notifiable diseases, leading to the first rank among parasitic diseases in Algeria (Mihoubi *et al*., 2012). During the last decades, clear evidence of increase in *Leishmania* transmission rate in Algeria was obtained, while some discrepancies between areas and clinical forms have been observed by local field workers.

The human visceral leishmaniasis (VL) of *Leishmania infantum* (MON-1 and *L.infantum* MON-24), conveyed by *Phlebotomus perniciosus*, is the most severe form of the leishmaniasis. It is an almost infantile affection in Algeria (Harrat *et al.*, 1992 ; Belazzoug *et al.*,1985 in Zait *et al.*, 2012). Mortality is observed in children from 0 to 4 years

old, in whom the incidence is 2.72 cases per 100 000 inhabitants (Fendri *et al.*, 2012). Therefore, it represents a real public health problem in Algeria, where there are approximately 540 new cases per year, meaning an incidence of 0,31 cases per 100 000 inhabitants. According to the National Institute of Public Health (I.N.S.P.), the incidence of human visceral leishmaniasis, leading to death in the absence of treatment (Mihoubi *et al.*, 2012), decreased from 0,72 in 2000 to 0,42 in 2001 (Zait *et al*., 2012). From 2003 to 2007, the I.N.S.P. (2007) estimated national incidence between 0,28 and 0,41 cases for 100 000 inhabitants.

Cutaneous leishmaniasis (CL) is presenting with three clinical forms in Algeria: (i) Zoonotic CL caused by *Leishmania major* (MON-25) and transmitted by *Phlebotomus papatasi*, (ii) sporadic CL of the North caused by *Leishmania infantum* (MON-24 *L.major* MON-80) and transmitted by *Phlebotomus perfiliewi*, and *Leishmania killicki* (MON-301), transmitted by *Phlebotomus sergenti* (Harrat *et al*., 2009). Cutaneous leishmaniasis burden was rapidly increasing during the last decade, with endemoepidemic mode : 4,450 cases were declared in 2000, 8,049 cases in 2002 , and 14,822 cases in 2004 , finally reaching a warning peak of alert with 30.227 cases in 2005. More recently, a significant decrease of reported cased started in 2006 to 7,784 cases in 2008 (Fendri *et al*., 2012).

The purpose of this work was to document the suspected decrease of *Leishmania* transmission in the Constantine's area, by extensive captures of female sandflies in the area, and parasite detection using real time PCR. Similar studies were designed elsewhere with mosquitoes natural populations (*Culex antennatus, Culex decens, Culex quinquefasciatus,* and *Mansonia uniformis* of Madagascar) to survey *Wolbachia pipientis* (Raharimalala, 2011) and *Wuchereria bancrofti* (Wijegunawardana, 2013). The real time PCR method was chosen because of its performances allowing parasite DNA detection, from the vector without requiring dissection of sandflies, limiting the risks of DNA dissemination and contamination.

2. Materials and Methods

Catches were located in areas where leishmaniasis cases have been recorded by the health services of Constantine in 2006 (Djezzar-Mihoubi, 2006). We have selected 10 sampling stations spread over 5 locations in the wilaya of Constantine: Hamma Bouziane, Didouche Mourad, Beni Hamidene, Zighoud Youcef and El-Khroub. The sampling was performed from 2011 to 2013 on sandflies natural populations during their emergence period (April to November), using miniature light traps type CDC (Center for Disease Control) **(Picture.1).** The traps are set up in biotopes favorable to sandflies development (stables built of stone with roofs made of thatch and steel, and shacks built of clay and stone with cracks). They are installed before sunset and remain functional throughout the night. The next morning, cages are detached and carefully closed while the engine is still running, taking care to prevent the escape of trapped insects. To immobilize the sandflies, the collection box is stored at -10°C for twenty minutes. Once dead, the insects are placed into tubes containing absolute alcohol.

Picture1: CDC's miniature bright traps.

2.1. Molecular diagnostic

2.1.1. DNA extraction

Leishmania DNA extraction was performed from captured females using the extraction kit QIAamp DNA Mini Kit (Qiagen, Germany) (Berdjane-Brouk *and al.*, 2012) according to the protocol described by the manufacturer: Twenty-five flies were placed in 1.5 ml Eppendorf tubes, and 180µl of ATL lysis buffer, then 20µl of Proteinase K, were added. Tubes were vortexed for 15 seconds and incubated in a thermostated water bath at 56 °C overnight. 200µl of ATL buffer and ethanol (100%) were respectively added and the tubes were incubated at room temperature for 5 minutes. After a brief centrifugation, the lysate was transferred to a column (placed on manifold identified at 2 ml tube) and centrifuged for 1 minute at 6000 g. AW1 (500µl) and AW2 (700µl) buffers and absolute ethanol (700µl) were added. After a 3 minutes centrifugation at 20,000g (in order to totally dry the membrane), the column was placed on a 1.5 ml identified sterile Eppendorf tube and incubated for 3 minutes at 56 °C. DNA was extracted after adding 30 µl of ATE, incubation at room temperature followed by centrifugation at 20,000 g. The DNA is then kept in Eppendorf tubes at -20 ° C.

2.1.2. Polymerase Chain Reaction

The real-time PCR of the genus *Leishmania* has been realized by Lightcycler according to SybrGreen I technology (Roche Diagnostics Ref.: 2239264), in capillary tubes, using the primers JW11 (5'-CCTATTTTACACCAACCCCCAGT-3') and JW12 (5'-GGGTAGGGGCGTTCTGCGAAA-3') selected on the conserved part of the DNA genes encoding the minicircles. These primers allow the amplification of the *Leishmania* genus (Nicolas *et al.*, 2002). In each series have been included: a negative control corresponding to non parasitized human blood and a positive control corresponding to the reference strain *L. infantum* (MOHM / TN / 80 / IPT1). The light intensity emitted by the fluorochrome, measured at the end of each amplification cycle is proportional to the amount of target DNA present in the sample. The identification of the PCR product is made in a post amplification melting phase by analyzing the specific fusion temperature of the amplified product. The PCR conditions are as follows: After denaturation for 4 minutes at 95° C, amplification takes place for 35 cycles with a denaturation step (95° C for 10 seconds), annealing (62° C for 10 seconds) and elongation (72° C for 10 seconds) (Djezzar-Mihoubi, 2006).
The real time PCR of species took place in the same conditions as the PCR genus but using the primers: JW13 (5'-ACTGGGGGTTGGTGTAAAATAGG-3 ') and JW14 (5'-TTTCGCAGAACGCCCCTACCC - 3') (Nicolas *et al.*, 2002) allowing the identification of species *L. donovani, L. infantum, L. major* and *L. tropica*. The reaction mixture is identical to that prepared for PCR genus. Are included in each series, a negative control (non-parasitized human blood), a positive control represented by the four reference strains: *L. donovani* (DD8), *L. infantum* (IPT 1), *L. major* (5ASKH) and *L .tropica* (K27) (Djezzar-Mihoubi, 2006).

3. Results and Discussion

This work aimed at the detection of *Leishmania* in a representative sample of the sandfly population of 1,498 females and 4360 males captured in the study area. The purpose was to assess the parasite load in a vector population in an area where leishmaniasis is endemic, in order to highlight the suspected local regression of this disease. It was expected to confirm the hypothesis advanced by Zait and Hamrioui (2009) and Fendri (2012) on the regression of leishmaniasis in the study area. Unlike previous studies whose objective was to identify, by RT-PCR, the species of *Leishmania* from the dissected vector (Boubidi, 2011; Berdjane-Brouk, 2012) in this study, we performed the DNA detection by RT-PCR without prior vector dissection.
The results showed that among the 60 performed PCR , only three were positive. The two species PCR performed on parasited female sandflies were negative due to the small amount of DNA present in leishmanian sandflies. This can be explained by the low abundance of infected sandflies in the surveyed communities. These results support the figures given by health services of Constantine. According to a retrospective study conducted at Constantine's Benbadis University Hospital on 892 cases diagnosed during public and private consultations, the evolution of visceral leishmaniasis from 2006 to 2010 showed a marked reduction from 13 cases in 2006 to five cases in 2010 (Fendri *and al.*, 2012). As for hospital services (Dermatology, Pediatrics and Infectious Diseases), 5 cases admitted in 2006, 2 in 2007, no cases in 2008, only 1 in 2009, no cases in 2010 and 4 in 2011 (National Institut of Public Health, 2006, 2007, 2008, 2009, 2010, 2011). Regarding cutaneous leishmaniasis, hospitalization services (Dermatology, Pediatrics and Infectious Diseases) have reported 14 cases hospitalized in 2006, 7 in 2007, 4 in 2008,

9 in 2009, 8 in 2010 and 7 in 2011 (National Institut of Public Health, 2006, 2007, 2008, 2009, 2010, 2011). Besides reducing cases of leishmaniasis in the region, statistics collected from the Central Pharmacy of the University Hospital show a significant decrease in the amount of cutaneous leishmaniasis treatment (Glucantime) given to various departments of the hospital between 2010 and 2013, and thereby strengthening the hypothesis of the regression of the disease in the region of Constantine **(Fig.1)**.

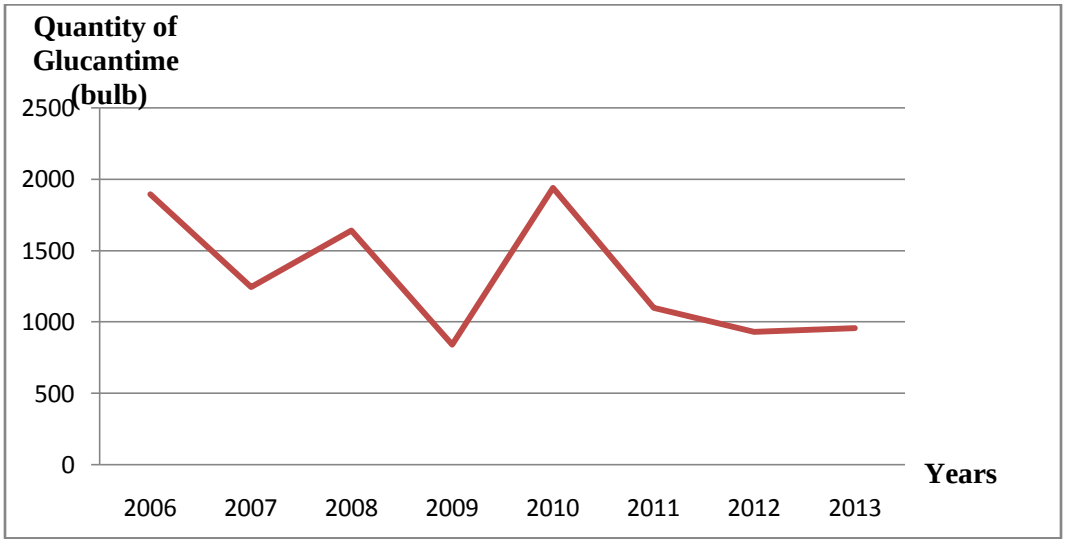

Figure 1: Amount of Glucantime treatment distributed to various departments of Constantine's Teaching Hospital between 2006 and 2013.

Despite the prevalence of the disease which remains high especially in rural areas, leishmaniasis is a clear downward trend in Algeria, notably in Constantine. In front of the alarming increase of leishmaniasis in the late 1990s, the extent of the geographical extension, the emergence of new outbreak regions and because of the heavy socioeconomic impact, Algeria urgently adopted a vector control strategy (spraying delthamethrine) materialized since 2004 (Achour Barchiche et Madiou, 2008; Zait and Hamrioui, 2009; Fendri *et al.*, 2012.). According to the National Institut of Public Health, the encouraging results are actually observed and the impact on the decrease in incidence is entirely confirmed (Zait and Hamrioui, 2009).

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