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Genotyping of Cutaneous Leishmaniasis and its association with CTLA-4 gene polymorphism

A Dissertation Submitted

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By

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1.1. Introduction

Various species of the genus *Leishmania* are responsible for the parasitic infection called Leishmaniasis. Leishmaniasis is the 3rd most significant arthropod-borne disease for the global burden of diseases (Kassebaum *et al.*, 2016). The World Health Organization (WHO) reported that various types of Leishmaniasis had been identified from 89 countries with over 350 million people at risk, with an estimated of 12–15 million subjects are affected worldwide. Two million new cases of Leishmaniasis are recorded yearly, of which 1.5 million with cutaneous Leishmaniasis (CL) (Torres-Guerrero *et al.*, 2017). Reports from WHO showed that over two-thirds of new cases of CL in 2015 happened in six countries comprising Iraq (Fakhar *et al.*, 2016).

The principal vectors of Leishmaniasis are the Phlebotominae sandflies. It is reported that there is a characteristic positive correlation between Leishmaniasis and the geographical distribution of sand flies. Sandflies prevail in dark places, near their breeding sites because they are weak flies (Akhlagh *et al.*, 2016).

Zoonotic CL and anthroponotic CL are the two kinds of CL prevalent worldwide. Anthroponotic CL is the non-rural or dry kind, while zoonotic CL is the rustic or wet kind. The causative agent for anthroponotic CL is *L. tropica*, while that of zoonotic CL is *L. major* (Salehzadeh *et al.*, 2014).

In Iraq, CL is prevalent across the whole country except in 3 northeastern provinces and is transmitted by *Phlebotomus papatasi* and *P. sergenti* species of sandfly (Salam and Azzi, 2014). Cases of anthroponotic CL predominantly occur in the suburbs of large cities, while zoonotic CL is more widespread in rural areas, especially in the northern and southern provinces of Iraq (Al-Warid *et al.*, 2017).

Beside parasitic load and environmental conditions, genetic factors of the host are significantly implicated in the acquisition and evaluation of the seriousness of CL (Handman *et al.*, 2005). Such a role becomes more prominent when the referred gene is involved in the immune response. T lymphocytes play a critical function in fighting against Leishmaniasis through cytokine production (T-helper cells) and destroying the parasite (Santos *et al.*, 2013). Interaction of CTLA-4 after its expression on activated T cells with CD80 and CD86 results in inhibition of T lymphocytes functions, CD80 and CD86 are ligands for CD28 (Rowsharravan *et al.*, 2018).

Pointing to the significant functions of CTLA-4 in T lymphocyte suppression, it seems that CTLA-4 plays a determinant function in inducing peripheral tolerance against self and foreign antigens. These are characteristic of self-tolerance and chronic infections, respectively (Brunner-Weinzierl and Rudd, 2018). Thus, genetic differences that result in up-regulation of this molecule could be linked to chronic infections and immune suppression, including CL. Past studies revealed that the CTLA-4 gene comprised various single nucleotide polymorphisms (SNPs) comprising CTLA-4 A/G at the +49 position (rs 231775) (Uzer *et al.*, 2010). This SNP was frequently reported to have a significant association with different diseases whether autoimmune (Wang *et al.*, 2017), cancer (Zhang *et al.*, 2018) or infectious (Hajilooi *et al.*, 2014). However, studies regarding the association of this polymorphism with CL are very rare or even absent, especially among Iraq patients. As there is no available vaccine against CL, finding such association can increase the current understanding of acquisition and severity of the disease and help in developing new drugs that target the immune system to prevent or overcome the infection.

Molecular characterization of the causative agent of CL may facilitate the determining molecular epidemiology and hence can help in introducing effective control programs. Polymerase chain reaction (PCR) brought about the infusion of techniques for identification and genetic characterization of *Leishmania* (El-Beshbishy *et al.*, 2013). Different genes such as 5.8S rRNA and internal transcribed spacer-1 gene have been used successfully for both diagnosis and molecular characterization of CL (Fakhar *et al.*, 2016). Studies concerning phylogenetic analysis and molecular characterization of *L. tropica* and *L. major* are scarce in Iraq despite the endemicity of the disease.

1.2. Aims of the study

- 1- To determinate the association between soluble CTLA-4 and occurrence and severity of CL
- 2- To determinate the correlation of CTLA-4+49G/A gene polymorphism with the incidence and severity of CL
- 3- To determinate The effect CTLA-4+49G/A gene polymorphism on serum level of soluble CTLA-4
- 4- To work the molecular characterization of local isolates of *L. tropica* and *L. major* and their relatedness to reference isolated from neighboring countries.
- 5- To determinate the function of soluble CTLA-4 in the pathogenesis of CL and detection of infection

2. Literatures Review

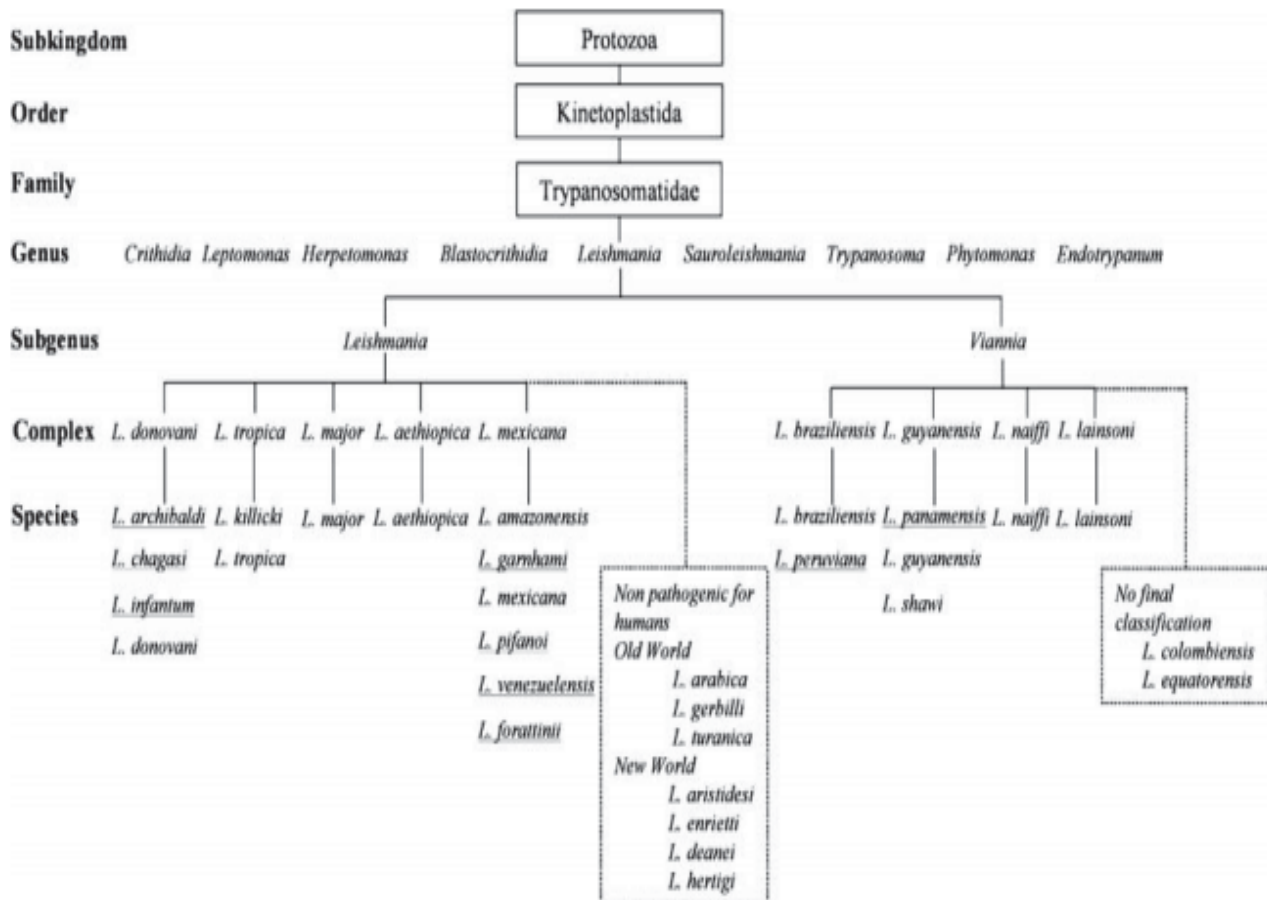
2.1. Leishmaniasis

Leishmaniasis is infection caused by a protozoa parasite of intracellular nature (genus *Leishmania*). It is transmitted when a female phlebotomine sandfly bites the infected person's skin(WHO 2014).

The clinical feature of leishmaniasis range from a self-resolving skin ulcer to a mutilating mucocutaneous infection and even to a potentially lethal systemic illness (Cheorghe *et al.*,2018).

2.2. Taxonomy

According to Banuls-Laure *et al.*, (2007) the classification of Species *L.tropica*, *L.major* as below:



2.3. Epidemiology

In the past two decades it has become obvious that leishmaniasis has expanded its range, in both tropical and subtropical areas (Haddad *et al.*, 2015), with diseases being present in new areas. This has engaged both known species and the present of new species (Karami *et al.*, 2013; Desbois *et al.*, 2014). Some of these are closely related species present in various regions around the world (Kwakye, 2016), In spite of these facts, leishmaniasis is remain one of the world's most neglected infections, affecting largely the poor population, mainly in developing countries (WHO, 2010).

There are predestined twelve million cases worldwide, and there are around one point five million cases of cutaneous leishmaniasis happen worldwide annually, and over two-third of CL new cases occur in countries: Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, Colombia and Peru. Death cases are rare since CL is normally a self-healing condition (Meireles *et al.*, 2017; Al-Sudany and Ali, 2016).

The infection present in epidemiological forms, zoonotic CL (ZCL), caused by *L. major* and usually occurs in rural regions where the main host is some species of mammals such as some rodentia and carnivora and *Phlebotomus papatasi* served as a vector. The second form of infection is anthroponotic CL (ACL) which is due to *L. tropica* in medium and large sized regions, where humans are the original reservoir host and *P. sergenti*, the original vector (Aflatoonian *et al.*, 2019).

Both ACL and ZCL were communicate as causative factors of leishmaniasis in Iraq. According to the results of many local reports, the disease epidemiologically is uneasy with major and uncertain fluctuations in the number of cases (Al-Qadhi *et al.*, 2015 ; Rahi *et al.*, 2014).

CL in Iraq was declined during the malaria control program and house spraying with Dichlorodiphenyltrichloroethane DDT, but when this was discontinued in the mid-1960s, the incidence was returned. In the period of the Gulf War in 1991, The peak incidence of CL reached 45/10,000 population in 2008 (Al-Obaidi *et al.*, 2016).

A maximum reported incidence rate was observed in 2015 and a minimum reported cases was observed in 2013 (Al-Obaidi *et al.*, 2016).

2.4. Risk factors

Several risk factors influencing host susceptibility to leishmaniasis some of these related to host which are represented by malnutrition, poor sanitary, age, and gender, geographical, seasonal distribution and immune status (Salam and Azzi 2014).

The disease increases mainly by increased exposure to infected sandfly. Number of this insect is affected by environmental condition such as building dams, widening areas under cultivation creating new irrigation schemes and migration from rural to urban areas (Ngere *et al* 2020)

2.5. Transmission

Leishmaniasis is transmitted through the bite of an infected female sand fly, the vector which is dependant on environmental conditions, and changes in environmental conditions result in changes in pattern of transmission, and as a result, CL may emerge in non-endemic regions. (Khan *et al.*, 2016; Majeed *et al.*, 2013).

Worldwide, vector-borne transmission is the major cause of transmission (Singh, 2006). The sand fly may acquire the parasite from a zoonotic reservoir. Laboratory, blood transfusion, congenital and sexual transmission are possible, and transmission by domestic or wild mammals has also been reported (Quinnell and Courtenay, 2009).

Summary

Background: The causative agents of Cutaneous Leishmaniasis (CL) are intracellular protozoan parasites of the genus *Leishmania*, mainly *L. tropica* and *L. major*. CL is a vector-borne disease. In endemic areas, only a proportion of exposed subjects under almost similar circumstances develop the disease, reflecting the role of genetic role in resistance and susceptibility to infection. Since cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is implicated in the repression of T cell proliferation, cytokine generation and cell cycle advancement, it is reasonable to postulate that single nucleotide polymorphism (SNP) in *CTLA-4 gene* can influence an individual susceptibility to CL.

Aims: This study aimed to investigate the association of CTLA-4+49G/A gene polymorphism with incidence and severity of CL, and the role of soluble CTLA-4 in the pathogenesis of CL and in detection of infection, as well as molecular characterization of local isolates of *L. tropica* and *L. major* and their relatedness to reference isolated from neighboring countries.

Subjects and Methods: This is a cross-sectional prospective study including a total of 110 patients with confirmed CL (60 newly diagnosed and 50 under treatment patients) as well as 60 age- and sex-matched healthy subjects as a control group. Dermal scraps were performed for each patients using sterile blade or dental broach from which the lesion exudates were collected in sterile tubes and impression smears were made on sterile slides. Further, about 5 ml of venous blood were collected from patients and controls. Each blood sample was divided in to two aliquots; 2 ml in EDTA tube and 3 ml in plain tube. DNA was extracted from the whole

When the sand fly bites the skin, they cause abrasions to the dermis of the skin due to their saw like, cutting edge shaped mouths. They form a pool through which the amastigotes get introduced, and later enter the blood stream when the fly sucks the blood.(Majeed B *et al.*, 2013).They suck not only blood but also damaged tissue of the dermis containing macrophages with amastigotes (Khan *et al.*,2016).

Leishmaniasis can spread through blood transfusion, and has been recorded in various countries including India. The parasite needs to be in the donor blood, as well as survive handling and storage in the blood bank, to infect the recipient. (Gonzalez *et al.*,2009;Rocha *et al.*, 2018).

Other study, majorly from animal models, suggest that this method of transmission is possible through contact with infected fluids (oral secretions and nasal, tonsilopharyngeal mucosa) (singh,2006).

2.6. Structure and Forms of *Leishmania*

The basic cellular architecture is defined by cross-linked sub-pellicular corset microtubules. This array continues throughout the cell life cycle, where cell division depends on the insertion and elongation of microtubules into the present array. The nucleus as well as the Golgi body, mitochondrion and a full set of organelles (single copy) are found inside the cell. The kinetoplast, which is the mitochondrial DNA directly linked to the basal body, is found anterior to the nucleus, and it is from that the flagellum extends (Wheeler *et al.*, 2016). An invagination in the cell membrane (known as the flagellar pocket) is found at the base of the flagellum. This pocket is the main site of endocytosis and exocytosis, and therefore serves as the link between the protozoan and its surroundings. (Lacomble *et al.*, 2009). Lipophosphoglycan (LPG) is a highly complex macromolecule composed of four distinct domains: a conserved glycosylphosphatidylinositol (GPI) anchor, a glycan core, a linear phosphoglycan chain (PG) and a terminating oligosaccharide cap. LPG