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Iran J Parasitol

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Iranian Society of Parasitology
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Original Article

In Vitro Study on Four Types of Commercial Lectins on *Leishmania infantum, L. major* and *L. tropica* with Stage-Specific Binding and Leishmania Species Identification

Sona Aghaee Haddadan [1], *Mehdi Mohebali [1,2], Homa Hajjaran [1], Abbas Rahimi Foroushani [3], Zahra Kakooei [1], Mohammad Javad Abbaszadeh Afshar [1,4], Zabihollah Zarei [1], Zahra Alizadeh [1], *Behnaz Akhoundi [1]

1. *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
2. *Center for Research of Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Epidemiology and Biostatics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
4. *School of Allied Medical Sciences, Jiroft University of Medical Sciences, Jiroft, Iran*

Received 10 Apr 2023
Accepted 16 Jul 2023

Keywords:
Leishmania;
Species identification;
Lectin;
Agglutination;
In vitro

***Correspondence**
Emails:
mohebali@tums.ac;
b-akhoundi@tums.ac.ir

Abstract

Background: We aimed to verify the susceptibility of *Leishmania infantum*, *L. major* and *L. tropica*, to commercial lectins in order to identify the three *Leishmania* species.

Methods: The degree of agglutination was determined both macroscopically and microscopically and was scored negative (-) to positive (from 1^{+}- 4^{+}) based on their percentage of agglutination.

Results: Jacalin and UEA-1 were capable of agglutination of *L. infantum* isolates in both logarithmic and stationary phases at a concentration of 1000 µg/ml (100%). *L. tropica* isolates showed agglutination with the lectin UEA-1 in both logarithmic and stationary phases (62.5% and 87.5%). *L. major* and *L. tropica* showed 75% agglutination with lectin Jacalin in both logarithmic and stationary phases. *L. tropica* isolates showed 25% agglutination with the lectin WGA in the logarithmic phase. *L. infantum*, *L. major* and *L. tropica* isolates showed 25, 12.5 and 37.5% agglutination in the stationary phase, however, did not show agglutination in logarithmic phases. *L. major* isolates showed 12.5% agglutination with the lectin PHA in the stationary phase, however, were incapable of agglutination with the *L. tropica* and *L. infantum* in both logarithmic and stationary phases.

Conclusion: Despite the fact, that JCA and I-UEA lectins were not able to completely separate *L. infantum*, *L. major* and *L. tropica*. WGA lectin and PHA lectin can help in separating the species of *Leishmania* parasites.

Introduction

Leishmaniasis is a tropical and subtropical disease characterized by significant clinical and epidemiological diversity. Leishmaniasis has a broad spectrum of clinical manifestations, including ulcerative skin lesions, destructive mucosal inflammation, and disseminated visceral infection (Kala-Azar) (1). The most common form is cutaneous leishmaniasis (CL), which can be caused by several *Leishmania* spp. and is rarely fatal. *L. tropica* is the causative agent of anthroponotic cutaneous leishmaniasis (ACL), while *L. major* causes zoonotic cutaneous leishmaniasis (ZCL). Visceral leishmaniasis (VL) is the most severe form of the disease and is usually fatal if left untreated. *L.* (*Leishmania*) *donovani* and *L.* (*Leishmania*) *infantum* are the two most important causative agents of human VL (1, 2).

Leishmaniasis remains a major public health problem despite advances in disease control and laboratory facilities to confirm and diagnose parasitic infections. This is due to not only environmental risk factors such as increased drug resistance, vectors' resistance to insecticides, massive migrations, and urbanization but also to individual risk factors such as HIV infection, malnutrition, genetic differences, etc. Leishmaniasis is a disease that still requires improved control strategies (1). *Leishmania* lesions commonly mimic other bacterial and fungal skin infections, and correct disease diagnosis and *Leishmania* species are essential for appropriate disease treatment, epidemiology, control, and prevention (3). Different approaches are taken to treating *L. tropica*, *L. major*, and *L. infantum*. In particular, for *L. tropica*, drug resistance and treatment failure are significant factors to consider.

Due to the diversity of disease reservoirs among species, knowing which species predominates in a given area is crucial for designing effective strategies for disease treatment, epidemiology, control, and prevention (4-6). Observing parasites in stained tissue smears and culturing tissue-derived promastigotes are laboratory diagnostic methods for cutaneous leishmaniasis (7). Although bone marrow and lymph node smears are the gold standards for diagnosing visceral leishmaniasis (8, 9), Other diagnostic methods including, Enzyme-Linked Immunosorbent Assay (ELISA), Indirect Fluorescent Antibody Test (IFAT), rK39, and Direct Agglutination Test (DAT) are widely used to diagnose visceral leishmaniasis (10, 11). All species and strains of the genus *Leishmania* are considered morphologically identical, and it is not possible to identify the species causing the leishmaniasis by the aforementioned diagnostic methods (12). Therefore, a simple and cost-effective method for distinguishing the species of the genus *Leishmania* is highly needed.

On the cell surface, many carbohydrate structures participate in intercellular and environmental interactions (13). Lectins are a wide class of proteins that bind to carbohydrates and are highly selective for the sugar chemical group and carbohydrate residues (14, 15). In addition, they serve a crucial role in regulating numerous normal and pathological processes in living organisms (15).

Lectins have frequently been used to investigate the surface membrane carbohydrates of various *Leishmania* strains to differentiate between pathogenic and nonpathogenic strains of this parasite (16).

In this study, we used *Artocarpus integrifolia* (generally called Jacalin), *Triticum vulgaris* (WGA), *Ulex europaeus 1* (UEA), and *Phaseolus vulgaris* (PHA) lectins to identify three *Leishmania* species, including *L. infantum*, *L. major* and *L. tropica*.

Materials and Methods

Parasite isolation and In vitro culture

Standard isolates of *Leishmania* parasites including, *L. tropica* (K27/74/SU/M and Mash-

had/04/IR/M isolates), *L. major* (MRHO/IR/75/ER and MHOM/IR/20/D12 isolates), and *L. infantum* (MCAN/IR/14/M14 and MCAN/IR/14/ Meshkin Shahr isolates) was provided by the Tehran University of Medical Sciences, Tehran, Iran.

L. infantum, *L. tropica,* and *L. major* promastigotes were obtained by planting the patient's bone marrow aspirates with visceral leishmaniasis and cultivating biopsy material taken from the skin lesion of a patient with cutaneous leishmaniasis into RPMI 1640 culture medium containing 10-20% heat-inactivated fetal bovine serum and penicillin/streptomycin (pen/strep) (100 unit /ml penicillin and 100 microgram/ ml streptomycin at 26 ˚C. The isolates were maintained in RPMI 1640 medium and harvested during the log phase of growth. In this study, logarithmic and stationary-phase parasites were obtained from cultures that had reached a constant density for 13 days, and then cells were separated by centrifugation for 15 min at 3000 rpm. Then cells were washed three times in cold phosphate-buffered saline (PBS) pH 7.2 or NaCl 0.9% buffer. Then, the number of promastigotes was counted per milliliter of culture medium using a Neubauer chamber. The obtained solution was diluted so that each well contained 10^8 cells/ml of promastigotes.

Lectin-agglutination test

We used commercially prepared lectins *Artocarpus integrifolia* (Jacalin), *Triticum vulgaris* (WGA), *Ulex europaeus 1* (UEA), and *Phaseolus vulgaris* (PHA). Qualitative testing of lectins and promastigotes was determined macroscopically in duplicate in 96-well microplates. Fifty µl of parasite suspension in PBS (10^8 cells) was mixed with an equal volume of PBS containing 1000 µg/ml lectin for 1 h at room temperature. The degree of agglutination was determined microscopically and was scored - to 4+ based on the size of cell agglutination and their proportion (14, 16).

Ethical approval

The study protocol was approved by the ethics committee of the Tehran University of Medical Sciences (IR. TUMS. SPH. REC.1398. 081).

Statistical analysis

All statistical analyses were performed using Version 25.0 of IBM SPSS (IBM Corp., Armonk, NY, USA). Values were presented as mean ± SD. We used the Mann-Whitney U test to assess the significance of the results. *P* value<0.05 was considered statistically significant**.**

Results

Agglutination of L. infantum isolates promastigote in logarithmic and stationary phase

Leishmania isolates were cultured In vitro, and all the isolates grew acceptably in RPMI 1640, reaching cell densities approximately 10^8 cells/ml.

Promastigote isolated as described above were incubated at 25˚C for 1 h with the agglutinins tested (Jacalin, UEA-1, WGA, and PHA). Only two commercial lectins, including Jacalin and UEA-1, were capable of agglutinating the *L. infantum* isolates in both logarithmic and stationary phases (100%). *L. infantum* isolates showed weak agglutination with the lectin WGA in the stationary phase (25%). PHA was not capable of agglutinating the *L. infantum* isolates in both logarithmic and stationary phases (Tables 1, 2).

Agglutination of L. major isolates promastigote in logarithmic and stationary phase

Only two commercial lectins (Jacalin and UEA-1) were capable of agglutinating the *L. major* isolates in both logarithmic and stationary phases (75% and 25% respectively). *L. major* D12 isolates showed a weak agglutination

with lectins WGA and PHA in stationary phase (12.5%, Tables 1, 2).

Agglutination of L. tropica isolates promastigote in logarithmic and stationary phase

Three commercial lectins, including Jacalin, UEA-1, and WGA, were capable of agglutinating the *L. tropica* K27 isolates in both logarithmic (75%, 50%, and 25%) and stationary phases (75%, 100%, and 25%). Rates of agglutination of *L. tropica* Mashhad isolates are 75%, 75%, and 25% in logarithmic and 75%, 75%, and 50% in stationary phase.

Lectin PHA was not capable of agglutinating the *L. tropica* in both logarithmic and stationary phases (Tables 1, 2).

Table 1: Agglutination activity of lectins for three cultured Leishmania species in logarithmic phase

Leishmania	*Artocarpus integrifolia (Jacalin) (%)*	*Ulex europaeus 1 (UEA-1) (%)*	*Triticum vulgaris (WGA) (%)*	*Phaseolus vulgaris (PHA) (%)*
***L. infantum* (M14)**	4+ (100)	4+ (100)	-	-
***L. infantum* (Meshkin)**	4+ (100)	4+ (100)	-	-
***L. major* (ER)**	3+ (75)	1+ (25)	-	-
***L. major* (D12)**	3+ (75)	1+ (25)	-	-
***L. tropica* (K27)**	3+ (75)	2+ (50)	1+ (25)	-
***L. tropica* (Mashhad)**	3+ (75)	3+ (75)	1+ (25)	-

*Agglutination was scored from - (no agglutination at 1000 µg/ml) to (+++) (virtually complete agglutination).

Table 2: Agglutination activity of lectins for three cultured Leishmania species in stationary phase

Leishmania strain	*Artocarpus integrifolia (Jacalin) (%)*	*Ulex europaeus 1 (UEA-1) (%)*	*Triticum vulgaris (WGA) (%)*	*Phaseolus vulgaris (PHA) (%)*
***L. infantum* (M14)**	4+ (100)	4+ (100)	1+ (25)	-
***L. infantum* (Meshkin)**	4+ (100)	4+ (100)	1+ (25)	-
***L. major* (ER)**	3+ (75)	1+ (25)	-	-
***L. major* (D12)**	3+ (75)	1+ (25)	1+ (25)	1+ (25)
***L. tropica* (K27)**	3+ (75)	4+ (100)	1+ (25)	-
***L. tropica* (Mashhad)**	3+ (75)	3+ (75)	2+ (50)	-

*Agglutination was scored from - (no agglutination at 1000 µg/ml) to (+++) (virtually complete agglutination).

Discussion

Leishmaniasis is one of the major neglected vector-borne tropical diseases transmitted through the bite of female phlebotomine sandflies (17). Depending on the *Leishmania* species, the reservoirs can be humans (anthroponosis) or different animals (zoonosis) (18). Most leishmaniasis cases of different species require different treatment regimens (19). Thus, identifying the species of *Leishmania* is essential for both the diagnosis and clarification of the disease's epidemiology.

Microscopic observation of parasites in stained tissue smears remains the gold standard of parasitological diagnosis, whereas unable to identify species (20). Recently, several molecular methods, especially those based on the polymerase chain reaction (PCR), have been developed to diagnose and identify *Leishmania* species (21). Molecular methods, despite being highly sensitive and specific, are used mainly in research studies due to complex implementation, expensive equipment, and materials (21). Therefore, aiming to distinguish *Leishmania* species, we studied the agglutination of *L. infantum*, *L. major* and *L. tropica* promastigotes by four commercial lectins, including Jacalin, UEA-1, WGA, and PHA.

Cell-surface carbohydrates of *Leishmania* play pleiotropic roles in parasite survival in the host, its infectivity and the digestive tract of the sand-fly vector (22). Surface lipophosphoglycan (LPG) is the major macromolecule of the promastigote stage of all *Leishmania* species and serves as the primary interface between the host and the parasite (23). Since different plant lectins, also named cell-agglutinating and sugar-specific proteins, can specifically recognize sugar molecules, they are widely used for the investigation/isolation and characterization of carbohydrates on cell surfaces (24). These carbohydrate-binding proteins can agglutinate cells In vitro (25). All the *Entamoeba histolytica* strains trophozoite contain lectin, which is an important virulence factor and could agglutinate a variety of different types of erythrocytes (26).

Merozoites of *Plasmodium falciparum* had lectin-like proteins on their surface that mediate the binding of erythrocytes and parasites bound to complementary carbohydrate determinants on target cell surface molecules (27). Lectins can be used to differentiate the Trypanosomatida of plants - *Phytomonas* sp. - from other lower trypanosomatids (28). In the present study, all the *Leishmania* isolates were agglutinated by three commercial lectins including *Artocarpus integrifolia* (Jacalin), *Triticum vulgaris* (WGA) and *Ulex europaeus 1* (UEA-1), with different degrees of agglutination and minimum concentration required to agglutinate. Our main findings were that Jacalin and UEA-1, could not distinguish between different species of *Leishmania* parasites. Among lectins, only Jacalin demonstrated significant agglutination activity with all *Leishmania* isolates in both logarithmic and stationary phases, therefore not suitable for identifying *Leishmania* species. In Andrade et al. study Jacalin showed agglutination activity with *L. tropica* and *L. major* isolates at a concentration of 125 µg/ml but *L. infantum* isolate did not react with this lectin at a concentration of 1000 µg/ml (16). In addition, Jacalin lectin was able to distinguish *L. donovani* complex from other *Leishmania* species (16).

We found that WGA can agglutinate *L. major* D12 isolate and *L. infantum* only in the stationary phase but *L. tropica* in both the logarithmic and stationary phases. Therefore, WGA can distinguish *L. tropica* from *L. infantum* and *L. major* in the logarithmic phase. These findings were in agreement with the results of Bandyopadhyay et al., which reported that WGA lectin could agglutinate *Leishmania* promastigotes in both logarithmic and stationary phases (29). WGA exhibited stage specificity in binding characteristics. In contrast, it was not in agreement with Dawidowic et al. findings (30).

We did not observe the binding of PHA lectin by *L. infantum*, *L. major*, and *L. tropica* in the logarithmic phase. Only *L. major* D12 isolate was poorly agglutinated in stationary-phase. Therefore, PHA can be used for *L. major* D12 isolate identification in the stationary phase. In the stationary phase, parasites show enhanced infectivity for cells and pathogenicity for their host. Therefore, PHA may also be used to purify infectious *L. major* promastigote.

Dawidowicz et al. reported that PHA lectin was incapable of agglutinating the promastigote and amastigote stages of *L. braziliensis* (31). In addition, no favorable outcomes with WGA lectin were observed.

Greenblatt et al. demonstrated that *Ulex europaeus* lectin identified *Leishmania* strains of the A serotype (32). In addition, UEA-1 lectin could agglutinate the Senegalese, Saudi, and Sudanese strains; therefore, it can be used for differentiating *Leishmania* strains (33). In agreement, binding of UEA-1 lectin with *Leishmania* species in both logarithmic and stationary phases was observed in this study; however, it could not differentiate the log and stationary phase promastigotes of *L. major* and *L. infantum*. It is noticeable that UEA-1 not useful for distinguishing *L. tropica* and *L. infantum*, *L. major* in the stationary phase.

The results of this study demonstrated that Jacalin lectin strongly agglutinated all the *Leishmania* isolates, and it not differentiated *L. infantum* from *L. major* and *L. tropica*. In addition, it cannot be useful for distinguishing logarithmic and stationary phase promastigotes of *L. infantum*, *L. tropica,* and *L. major*. Our findings diss-agree with Andrade et al. where Jacalin was the only lectin capable of distinguishing the *donovani* complex (16).

Conclusion

Lectin-mediated agglutination could be useful, rapid and easy for distinguishing *Leishmania* species but none of the four types of commercial lectins used in this study has been able to completely separate *L. infantum*, *L. major* and *L. tropica*. JCA and I-UEA were not able to completely separate *L. infantum*, *L. major* and *L. tropica*. WGA lectin by agglutinating of the promastigotes of the logarithmic stage of *L. tropica* and PHA lectin by agglutinating of the promastigotes of the stationary stage of *L. major* can help in separating the species of *Leishmania* parasites.

Acknowledgements

This study was approved and financially supported by Center for Research of Endemic Parasites of Iran, Tehran University of Medical Sciences (TUMS) (Project No: 98-01-160-41567).

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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