# PURIFICATION OF METACYCLIC LEISHMANIAL PROMASTIGOTES BY USING PEANUT LECTIN AGGLUTININ TEST .

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## ABSTRACT

*Lieshmania* metacyclic promastigotes are transmitted during blood meals after development inside the gut of the sandfly vector. The isolation from axenic cultures of procyclic and metacyclic promastigotes by peanut lectin agglutination followed by differential centrifugation is controversial in *Leishmania major* . The purpose of this study was to isolate both fractions simultaneously from the same population in stationary phase of axenic culture. We have confirmed the increased infection rate of PNA− promastigotes by inoculation in BALB/c mice infection experiments. These data support that metacyclic promastigotes are related with infectivity and the lack of agglutination with PNA is a phenotypic marker for this subpopulation.

## INTRODUCTION

Vertebrate represent the first host for *leishmania* multiplication. During this stage, *leishmania* have the ability to multiply inside the macrophages to amastigotes. In contrast, sandflys and in particular their midgut is the main host for *leishmania* multiplication to promastigotes extracellularly [(1)].

It has been documented that the leishmanial virulence their hosts such as mammalian and sandfly vector is due to glycoconjugates. The vast majority of pathogenic *Leishmania* species and especially in human have the ability to express glycolipid lipophosphoglycan (LPG). Similarly, glycosylphosphatidylinositol (GPI)-anchored proteins and proteophosphoglycans (PPGs) are expressed in *Leishmania*

promastigotes. These factors are potentially linked to the infection which be caused by *Leishmania* species[2]. All these factors together, from *Leishmania* transformation inside their hosts to the expression of virulent factors are crucial to mediate Leishmanial pathogenicity in its host [3].

Agglutination main features along with the Arachyshypogaea lectin, the peanut agglutinin (PNA) have been considered for the isolation process of metacyclic promastigotes. Agglutinating process of procyclic promastigotes mainly depends on a binding process which are can be performed between lectin and LPG galactose residues. It's well documented that the agglutinating properties could impaired by arabinose which is expressed during the metacyclogensis. Procyclic (Pro-PNA+) and metacyclic (Pro-PNA−) promastigotes which are agglutinating and non-agglutinating respectively can be isolated via centrifugation in axenic culture and especially in the stationary phase[4].In this study, we explored the metacyclic promastigotes and infective stages and we defined in details the main differences in lectin-binding molecules during the amastigote to promastigote conversion.

## MATERIAL AND METHODS

**Samples collection:**

All samples were collected from patients who were admitted to Tikrit hospital in Salah AL-Deen province. Basically, those patients were infected with *Leishmania* and potentially diagnosed by professional physicians in dermatology unit at the same hospital. The lesion place had been cleaned with a cotton soaked in 70% ethanol or isopropanol to disinfect the lesion edges. 1 ml syringe with a short needle (20 gauge) was used to inject PBS to the lesion site. Once the needle is inserted, it was twisted one to three times subcutaneously. This procedure was carried out to make sure some tissues were came off the lesion. Finally, BPS containing tissues derbies and exudates were taken off the lesion by the syringe and placed straight away in special media is used to grow Leishmania[5, 6].

**Media preparation:** samples of *L.major* isolates were inoculated in Roswell park memorial institute medium RPMI 1640 L-glutamine (Sigma,St.Louis). Other supplements such as FCS 10%, as a source of protein, and Penicillin 1000U/ml (0.00598 gm) ,0.3mg/ml streptomycin and nystatin 250 U/ml(0.000514gm), to avoid cross contamination, had been added to this media[7]. The tubes used in this study

were subjected to incubation at 25 ºC for 21 days. A clean slide was used to test the cultured smear. A little drop from the growth smear was taken and placed on that clean slide and was lefted for at least one minute to dry. Giemsa stain then applied to the smear drop and was lefted for 20-25 mins then washed off with DW and tested under the microscope by a power of 100A[5].

**Agglutination assays:**

Parasites were plated out in 96 well plates in peanut agglutinin (PNA) media (Sigma) in 1-2x$10^8$ parasites/ml. In contrast, 50 µg/ml of peanut lectin agglutinin was used. Hanks balanced salt solution (HBSS) without NaHCO3 was used along with lectin in HBSS to provide a source of balance to parasite suspension at room temperature for 30 mins. For promastigotes counting, hemocytometer was used to figure out the actual number of promastigotes. PNA-agglutinated and unagglutinated promastigotes were subjected to HBSS three times to remove any suspended particles. Moreover, the promastigotes was suspended with 2 x $10^8$ to 5 x $10^8$/ml HBSS simultaneously with 100 µg of PNA per ml. Then the suspension was incubated for 30 mints and centrifuged at 150g for 5 mins. The unagglutinated parasites were suspended in the supernatant which was taken off carefully to avoid loosing any agglutinated promastigotes and the rest, agglutinated ones, in the tubes were washed twice and counted [8].

**Mouse infections**:

Male mice aged 4-6 weeks are sourced from the college of veterinary medicine-Tikrit university were used in this study. These mice were grouped to four groups each one of five mice, the first group injected with 100%agglutinated peanut (PNA+) ,second group injected with the un agglutinated peanut (PNA-),the third group with purified PNA and the last group stay without injection as a control. The mice injected in the left hind footpad subcutaneously with $10^6$ or $10^7$ promastigotes diluted in HBSS. Footpads were measured with a direct-reading vernier caliper.

## RESULTS

As shown in fig.1, Giemsa stained smears of promastigotes isolated in RPMI 1640 media from cutaneous leishmaniasis patients after incubation in 25 ºC for ,12,21 days ,examined at
40X.

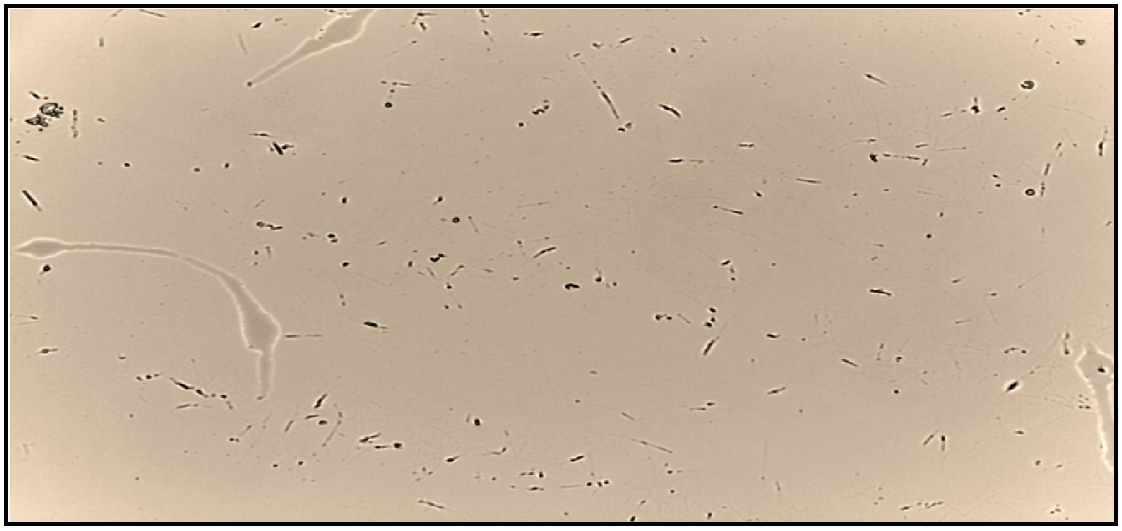


**Fig (1):** promastigotes under 40X light microscope stained with Giemsa stain after 6days inculcated in RPMI 1640 in 25ºC .

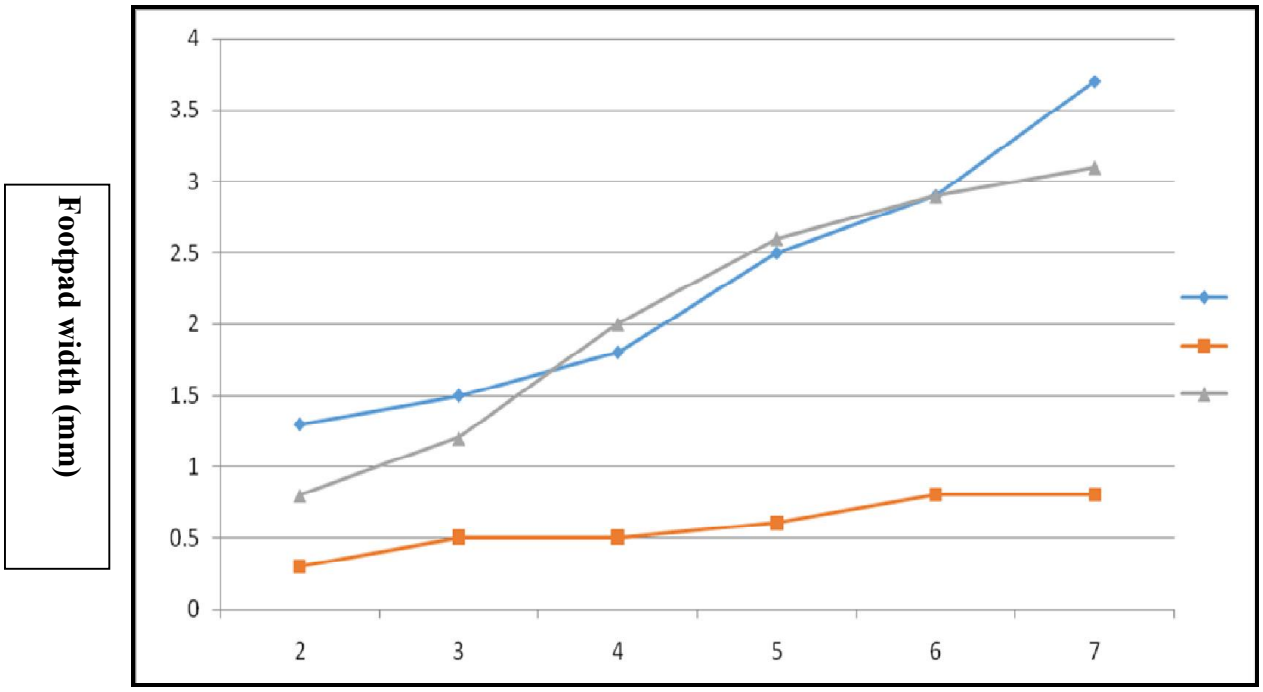
Fig 2,shows the agglutination as a result of mixing 1-2 x $10^8$ parasites per ml and 50 µg/ml of peanut lectin agglutinin(PNA),from 6,12 days after incubation .as shown there were two pattern of reactions as PNA+ which represent procyclic (Pro-PNA+) and non-agglutinating metacyclic (Pro-PNA−) promastigotes within the stationary phase of culture.

Fig (2): Agglutination with Peanut lectin agglutinin (PNA), differential centrifugation procedure to isolate the agglutinating procyclic (Pro-PNA+) and non-agglutinating metacyclic (Pro-PNA−) promastigotes within the stationary phase of culture.

In fig (3),the animals inoculated with PNA+ ,PNA-,and purified PNA-, those inoculated with PNA+ have no cutaeous lesion shown at the site of inoculation while cutaneous lesions found in the groups inoculated with PNA- and purified PNA-, in all infected mice started with redness and swelling at the site of inoculation in the 21-28 days post-infection swelling increased progressively onwards, at least half of the animals had developed characteristic infiltrations in the skin at the back of the mice, Lishmania were found in smears taken from some animals.

Fig (3): footpad swelling and increase in width after inoculation of PNA-and purified PNA- in BALB/c mice after weeks of inoculation .

In Fig (4) the *in vivo* virulence patterns of promastigote populations defined on the basis of PNA agglutination is shown. Promastigotes derived from logarithmic-phase cultures of isolate , which were 100% PNA+ , were relatively avirulent for BALB/c mice. A promastigote inoculum derived from a day 6 stationary-phase culture, produced detectable lesions by week 4. When PNA- promastigotes were purified from these same stationary-phase cultures, this inoculum was extremely virulent, producing detectable lesions by the end of week 2, so that by week 7, the loss of the infected footpad had already occurred in most mice.




FIG(4):. Course of lesion development in BALB/c mice inoculated with day 6 stationary-phase (▲), day 12 stationary-phase (■) or PNA- purified (♦) promastigotes from leishmania isolates . All mice were inoculated with $10^6$ promastigotes .

## DISCUSSION

Metacyclic promastigote along with procyclic promastigote, the main transmissible stages by *Leishmania* first host sand fly, have been considered in this study. The mentioned stages are the clue to understand in depth the virulence of *Leishmania*. The process of promastigotes transformation from non-infective to infective stage could give us a hint how it's important in their hosts i.e midgut of sand-fly and axenic culture [(1)]. Interestingly, our *in vitro* studies showed that the metacyclic promastigotes of *Leishmania major* can be recognized from other non-infective ones by their ability to loss their agglutination properties in an especial lectin PNA concentrations.

Moreover, *in vivo* results in this study shows that PNA agglutination and non-agglutination virulence could be ameaningful biomarkers for metacyclic promastigotesof *L. major.*In conjunction with, studying these markers can be beneficial to emphasize the divergence between metacyclogenesis which involvesin *Leishmania species* during the process of growing and multiplication. Lectins, defined

as carbohydratebindingproteins or glycoproteins , have been used in theidentification of exposed surface glycoconjugates because oftheir ability to noncovalently and relatively bindto saccharide residues[9].

Figure 4 shows the virulence of two different patterns and especially after 6 days as an early stage and after 12 days as a late stage. *In vivo* results in this study revealed that after 6 days stationary phase, promastigotes were potentially virulent and consequently induced footpad lesion in about two weeks after infection. In contrast, 12 days stationary promastigotes weren't necessarily virulent and these promastigotes showed complete recovery after their isolation from mice footpad lesion. In addition, our *in vivo* data in this study pointed out that promastigotes isolated from agglutinated peanut lectin were completely avirulent and for that reason these promastigotes didn't produce any footpad lesion in mice even after 4 months of infection. This incompatibility in promastigote metacyclogenesis could reflect their capability to promote infection.

It seems from what mentioned above that the two different developing stages of *Leishmania major* are capable in producing infection if they were included in the inoculum. Promastigotes agglutination ability with the PNA should be considered in understanding the pathogenicity of *L. major*. In summary, we conclude that the biomarkers which are crucial for metacyclic promastigotes of *Leishmania major* and other species can be extrapolated.

## تنقية metacyclic promastigotes للشمانيا باستخدام اختبار تلازن لكتين الفول السوداني

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### الخلاصة

تنتقل ميتاسايكليك بروماستيكويت للشمانيا *Lieshmania* metacyclic promastigotes خلال تناول وجبة الدم بعد تطورها داخل الأمعاء لذبابة الرمل الناقلة. ان عزل procyclic promastigotes وmetacyclic promastigotes من المزارع النقية للطفيلي بواسطة تلازن لكتين الفول السوداني ويتبعها

عمل طرد مركزي تفاضلي لها هو امر مثار للجدل في *Leishmania major*. ان الغرض من هذه الدراسة هو عزل كل اطوار الشمانيا من طفيليات اللشمانيا النامية في المزرعة النقية بشكل اني في مرحلة الثبات. وقد أكدنا على زيادة معدل العدوى بطور PNA-promastigotes عن طريق حقن العدوى في فئران التجارب من نوع BALB/c. ان هذه البيانات تدعم أن metacyclic promastigotes له علاقة بالعدوى بالشمانيا وان عدم تلازنها مع لكتين الفول السوداني (PNA) هو علامة مظهرية لهذا الطور من طفيليات اللشمانيا.

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