

Research Article

PGF_{2α} Synthase-Like Proteins are Expressed in Promastigotes of Old World *Leishmania* Species but not in New World Species

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Background: Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania* and spread by the bite of infected sand fly species. The disease is characterized by an increase in prostaglandin production in the host. PGF_{2α} is among the prostaglandins that are synthesized by *Leishmania* sp.

Objectives: To compare the expression profiles of PGF_{2α} synthase-like proteins in Old and New World species of *Leishmania* so as to provide insight into the role of these proteins.

Methodology: To detect gene expression at transcription level, polymerase chain reaction was carried out using *L. major* PGF_{2α} synthase gene specific primers and cDNA from *L. major*, *L. donovani*, *L. tropica*, *L. amazonensis*, *L. braziliensis*, *L. mexicana* and *L. chagasi* promastigotes. To detect expression at translation level, total protein from promastigotes of the above parasites was analyzed on a Western blot using *T. brucei*-specific rabbit anti-PGF_{2α} synthase polyclonal antibodies.

Results: At the transcription level, PGF_{2α} synthase gene expression was detected in Old World species *L. major*, *L. donovani* and *L. tropica*, but was absent in the New World *L. amazonensis* and *L. mexicana*. It was expressed at low levels in the New World *L. chagasi*. Western blot analysis confirmed the presence of PGF_{2α} synthase - like proteins in Old World and not in New World species.

Discussion: These findings suggest that New World *Leishmania* may have evolved new ortholog genes to produce PGF_{2α}. Alternatively, the ancestral PGF_{2α} synthase gene may be present in the New World species but has mutated or been lost due to speciation during evolution.

Key words: Prostaglandins; PGF_{2α} synthase; *Leishmania*

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1. Introduction

The *Leishmania* parasites cause a wide spectrum of disease that varies in symptoms and clinical manifestations depending on the infecting species and host immune status (Cunha et al, 2013). This parasite exists as an intracellular amastigote in vertebrate hosts or a promastigote found in sandfly vectors (Singh,

2006). Leishmaniasis is prevalent throughout the tropical and sub-tropical regions of Africa, Asia, the Mediterranean, Southern Europe (Old World) and South and Central America (New World). This disease is endemic in 98 countries with more than 350 million people at risk and an estimated 1.3 million new cases and 20 000 to 30 000 deaths annually (WHO, 2014).

Production of prostaglandins (PGs) increases during infection of the host by *Leishmania* parasites (Gregory et al, 2008) suggesting a major role by these molecules in the pathogenesis of the disease. Prostaglandins exacerbate the outcome of infection with *Leishmania* in mice by inhibiting activation induced cell death of T helper 2 (Th2) cells (Kaul et al, 2012) suggesting a role for PGs in the pathogenic mechanisms of these organisms. Studies indicate that PGE₂ inhibits the T helper 1 (Th1) response and promotes the Th2 response by suppressing the natural killer cell induced IFN- γ synthesis (Walker & Rotondo, 2004). Furthermore, PGE₂ can shift the balance of CD4⁺ helper T cells towards a Th2 type immune response (Kuroda et al, 2000) thereby playing a role in the inability of animals to resolve infections. Thus, the inability of the host to develop an effective immune response involves PGs (Reiner & Malemud, 1985).

Prostaglandin production has been studied in other kinetoplastids including *Trypanosoma brucei*. *T. brucei* synthesizes PGD₂, PGE₂ and PGF_{2 α} from endogenous or exogenous stores of arachidonic acid (AA). Among these prostaglandins, PGF_{2 α} is the major prostanoid synthesized by *Leishmania*. Since the production of prostaglandins increases during infection of the host by *Leishmania* parasites (Gregory et al, 2008), increased levels of PGs in leishmaniasis may be due to activity of PGF_{2 α} synthase-like enzymes in those parasites. Prostaglandin production in kinetoplastids *Leishmania*, *T. brucei* and *T. cruzi* parasites is not inhibited by aspirin and indomethacin (Kabututu et al, 2003, Kubata et al, 2000, Kubata et al, 2002), yet these classical non-steroidal anti-inflammatory drugs (NSAIDs) prevent formation of prostanoids by inhibiting mammalian cyclo-oxygenase activity (Wilson et al, 2004). Thus, the pathway for production of prostaglandins in *Leishmania* is distinct from the mammalian counterpart and presents a potential drug target for the treatment of *Leishmaniasis*. We sought to investigate differences between Old World and New World *Leishmania* species by investigating the expression profiles of PGF_{2 α} synthase-like enzymes in these parasites.

2. Materials and Methods

2.1 *Leishmania* parasites

Isolates of *L. major* (ATCC 50122), *L. donovani* (ATCC 50127), *L. tropica* (ATCC 30012), *L. amazonensis* (ATCC 50131), *L. braziliensis* (ATCC 50135), *L. mexicana* (ATCC 50157) and *L. chagasi* (ATCC 50133) promastigotes were obtained from the cryobank of American Type Culture Collection (Manassas, VA, USA). These were grown in Schneider's Insect Medium (Sigma™) containing 20% heat inactivated fetal bovine serum (Hyclone laboratories, Logan, USA), supplemented with 100 U/ml penicillin, 10mg/ml streptomycin sulfate and 25 μ g/ml amphotericin B (Sigma-Aldrich, St. Louis, USA) at 25°C. The promastigotes were harvested by centrifugation at 1200 x g for 10 minutes, at 4°C to obtain cell pellets.

2.2 PCR amplification and nucleotide sequencing

Total RNA (tRNA) was extracted from the cell pellets (10⁶ cells) of each of the *Leishmania* species above using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according

to the manufacturer's instructions. 1 μ g of RNA from each species was used to perform first strand cDNA synthesis using 100 units of Superscript II reverse transcriptase and 0.5 μ g Oligo dT primers (Invitrogen, Carlsbad, CA, USA) in a total reaction volume of 20 μ l. The reaction mixture was incubated at 42°C for 50 min followed by inactivation at 70°C for 15 min. PCR was carried out using the first strand cDNA from each of the *Leishmania* species as template. The mixture contained 1 μ l of cDNA from each *Leishmania* species as template, Ex Taq buffer (Takara Bio Inc. Otsu, Shiga, Japan), 2.5mM dNTP mixture, 0.5 μ M sense and antisense primers, 1.5U Ex Taq polymerase (Takara Bio Inc. Otsu, Shiga, Japan) and distilled water to give a total reaction volume of 25 μ l per species sample.

To amplify the PGF_{2 α} synthase gene in the seven *Leishmania* species, *L. major* prostaglandin F Synthase (*LmPGFS*) gene specific primers (Kabututu et al, 2003) were used. Cycling parameters included an initial denaturation at 95°C for 1 minute, followed by 35 cycles each of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute. These were followed by a final extension of 72°C for 10 minutes. The resulting PCR amplicons were separated by electrophoresis in 1% agarose gel (Takara Bio Inc. Otsu, Shiga, Japan) and stained with 0.5 μ g/ml ethidium bromide (Sigma-Aldrich, ST. Louis, USA), before visualization under UV light in a AlphaImager gel imaging system (Alpha Innotech corp, San Leandro, USA). The PCR amplicons were purified using GFX PCR DNA and Gel Band purification Kit (GE Healthcare, Pittsburgh, USA) according to the manufacturer's instructions.

Nucleotide sequences were determined using the Sanger dideoxy chain termination chemistry (BigDye Terminator) on the Applied Biosystems 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequence data was used to characterize the encoded genes. Search for similar genes to the one encoded by the sequences was performed on the Genbank database at the National Center for Biotechnology Institute (NCBI) using the Basic Local Alignment Search Tool (BLAST; (Altschul et al, 1990).

2.3 Lysis of promastigotes and determination of protein concentration

Leishmania promastigote cell pellets (10⁶ cells) were ruptured by hypotonic lysis using double distilled water containing the Complete™ cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany). Soluble proteins were obtained in the supernatant after centrifugation at 3000 x g for 15 minutes, at 4°C. Total protein concentration in the cell lysates was determined using Pierce bicinchoninic acid (BCA) assay with Bovine serum albumin (BSA) as a standard according to the manufacturer's protocol (Pierce Chemical Co., Rockford, IL, USA).

2.4 Western Blot analyses of PGFS

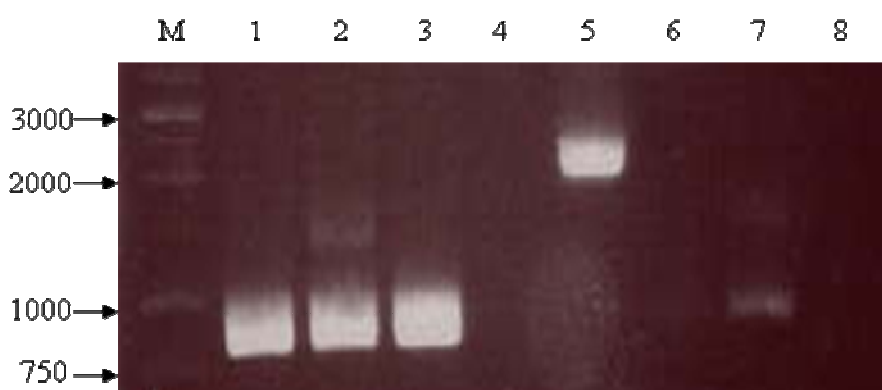
Western blot analyses of the PGFS were carried out as previously described (Kabututu et al, 2003). Briefly, 25 μ g of *Leishmania* promastigote lysates from each of the 7 species were resolved on 13% (w/v) SDS-PAGE gels. Separated proteins were transferred to an Immun-

Blot polyvinylidene difluoride (PVDF) membrane (BIO-RAD Laboratories, Hercules, CA, USA) at 200mA for 1 hour before blocking with 5% non fat dried milk (Kenya Highland brand) in Tris Buffered Saline containing 0.05% (v/v) Tween-20 (TBST) at 4°C overnight. The membrane was then incubated at 4°C with rabbit anti-*T. brucei* PGF_{2α} synthase polyclonal antibody (obtained from Department of Molecular and Behavioral Biology, Osaka Bioscience Institute, Osaka, Japan) applied in 5% non fat dried milk (Kenya Highland brand) and TBST. The membrane was then washed thrice in TBST, and incubated in horseradish peroxidase conjugated donkey anti-rabbit IgG (GE Healthcare, Pittsburgh, USA) at a concentration of 10μg/ml for 1 hour at 4°C. The membrane was washed thrice with TBST at 4°C, and the bound antibodies detected by enhanced chemiluminescence (ECL) reagents for Western blot detection according to the manufacturer's instructions (GE Healthcare, Pittsburgh, USA).

3. Results

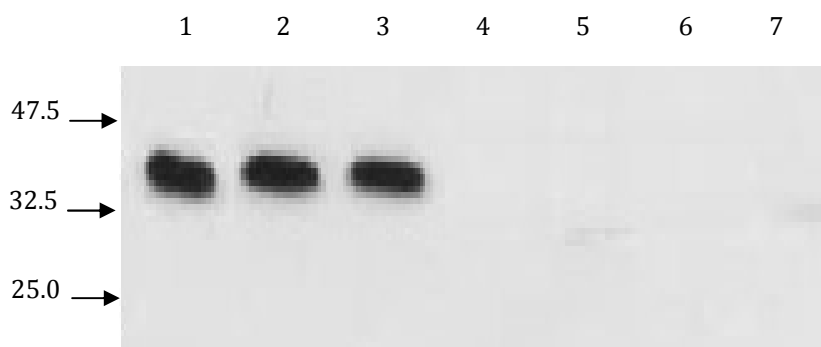
3.1 PGF_{2α} synthase-like transcripts are present in all Old World and only one New World species of *Leishmania*

A prominent 855-bp amplicon corresponding to the PGF_{2α} synthase was amplified and detected from the cDNA of all Old World species including *L. major*, *L. donovani* and *L. tropica* (**Figure 1**, lanes 1, 2 & 3). However, a less prominent band of the same size was detected using the cDNA in a single New World species, *L. chagasi* as the template (**Figure 1**, lane 7). There was no amplification for the other two New World species, *L. amazonensis* and *L. mexicana* (**Figure 1**, lanes 4 and 6). Surprisingly, a prominent 2kb fragment was amplified when cDNA from *L. braziliensis* was used as the template (**Figure 1**, lane 5).



The PCR amplicons were run on a 1% agarose gel. M = Promega 1-Kb ladder; lane 1 = *L. major*; lane 2 = *L. donovani*; lane 3 = *L. tropica*; lane 4 = *L. amazonensis*; lane 5 = *L. braziliensis*; lane 6 = *L. mexicana*; lane 7 = *L. chagasi*; lane 8 = negative control without cDNA

Figure 1: RT-PCR analysis of PGF_{2α} synthase gene expression from *Leishmania* species.



Proteins were transferred from the polyacrylamide gel to PVDF membrane (BIO-RAD Laboratories). Rabbit anti-*T. brucei* PGF_{2α} synthase polyclonal antibody was used as the primary antibody while 10μg/ml horseradish peroxidase conjugated donkey anti-rabbit IgG (GE Healthcare) was used as the secondary antibody. The signal on the membrane was visualized using ECL reagents (GE Healthcare). Lane 1 = *L. major*; lane 2 = *L. donovani*; lane 3 = *L. tropica*; lane 4 = *L. amazonensis*; lane 5 = *L. braziliensis*; lane 6 = *L. mexicana*; lane 7 = *L. chagasi*.

Figure 2: Western blot analysis of protein lysates to detect PGF_{2α} synthase in *Leishmania*.

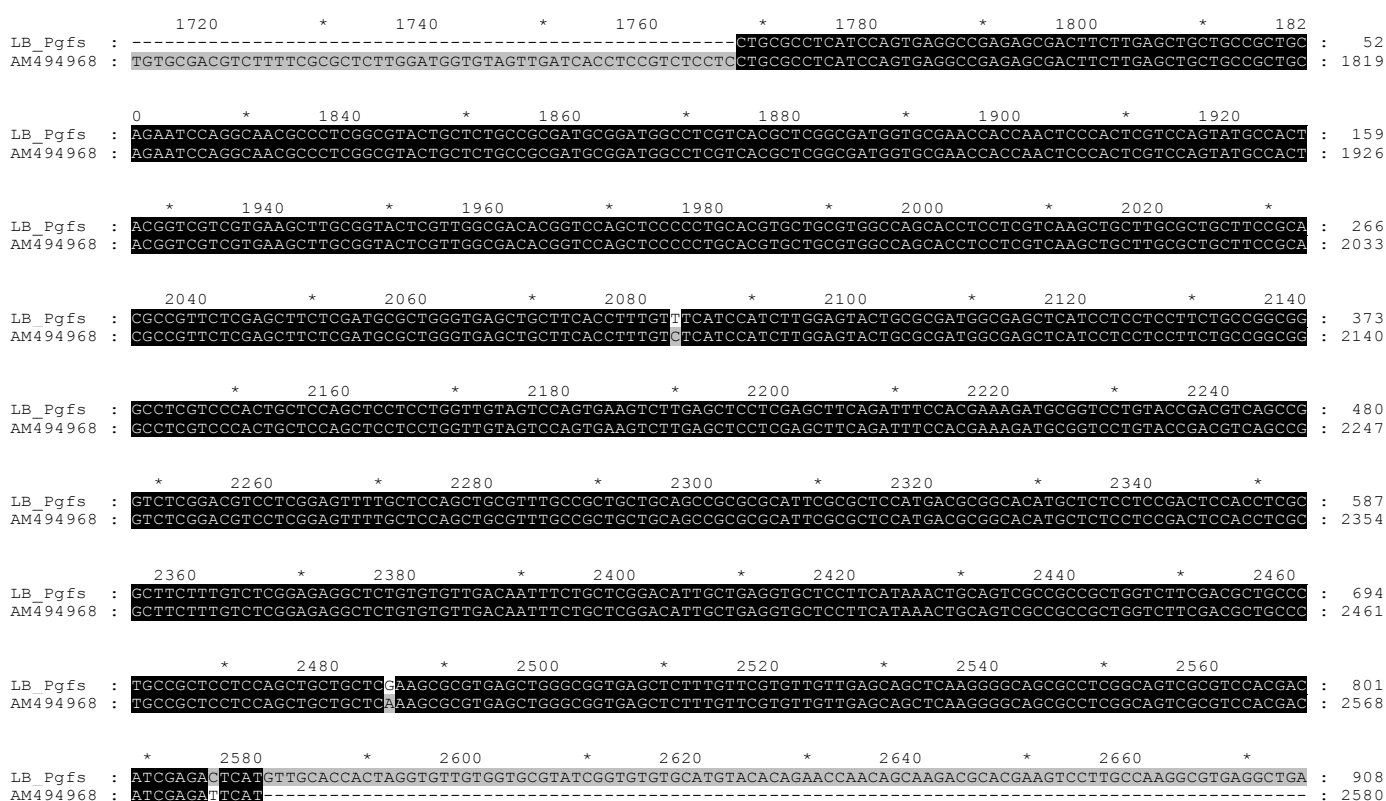
3.2 Western Blot analysis of *Leishmania* protein lysates

Rabbit anti-*T.brucei* PGF_{2α} synthase polyclonal antibody detected a 34-kDa immunoreactive protein in lysates derived from the Old World species, *L. major*, *L. donovani*, and *L. tropica* (Figure 2; lanes 1-3). No PGF_{2α} synthase protein was detected in the New World species of *L. amazonensis*, *L. braziliensis*, *L. mexicana*, *L. braziliensis* and *L. chagasi* (Figure 2; lanes 4-7).

3.3 Sequence analysis of *L. major*, *L. donovani*, *L. tropica* and *L. braziliensis* PGF_{2α} synthase genes

Homology search against the NCBI's non-redundant database and sequence analysis of the PCR products of *L. major*, *L. donovani* and *L. tropica* revealed that the

nucleotide sequences showed the highest similarity to *L. major* (Acc. No. XM_001685307.1), *L. donovani* (Acc. No. XM_003863417.1) and *L. tropica* (Acc. No. AB079546.1) prostaglandin F synthase in the database. Homology search of the *L. braziliensis* product showed that indeed the nucleotide sequence belonged to *L. braziliensis*. It showed the highest similarity to a conserved gene of *L. braziliensis* MHOM/BR/75/M2904 coding for a partial mRNA of a hypothetical protein, found on chromosome 31 (Acc. No. AM494968). Pairwise nucleotide sequence alignment was carried out to compare the *L. braziliensis* gene fragment and AM494968 (Figure 3). The *L. braziliensis* nucleotide sequence lies between position 1768 and position 2580 on the *L. braziliensis* partial mRNA on chromosome 31. However, further analysis revealed that it was not a PGF_{2α} synthase gene, but a non specific PCR product as a result of mispriming.



The AM494968 nucleotide sequence had the highest bit score in the BLASTn analysis and was fetched from GenBank database prior to this analysis. The pair-wise alignment was obtained using MUSCLE version 3.8 and applying default parameters of the program.

Figure 3: Pair-wise nucleotide sequence alignment of the putative *L. braziliensis* PGF_{2α} synthase gene fragment (LB_Pgfs) and the partial mRNA of a hypothetical protein found on *L. braziliensis* chromosome 31 (AM494968).

4. Discussion

Using RT-PCR and western blot analyses, this study shows that PGF_{2α} synthase - like gene is transcribed in Old World species *L. major*, *L. donovani* and *L. tropica* but not in two of the New World species *L. amazonensis*, and *L. mexicana*. The gene is however transcribed in *L. chagasi* but at low levels. The detection of transcription of PGF_{2α} synthase - like gene in Old but not in most New World species of *Leishmania* suggests that New World

Leishmania may have evolved orthologous genes to produce PGF_{2α} or that ancestral PGF_{2α} synthase gene in the New World species have evolved in a paralogous manner due to speciation. This is probable since the Old and New World parasites occupy different ecologic niches. *Leishmania* and other parasites are believed to have originated in Africa and were transported to Europe, Asia and the Americas during migration of man and animal reservoirs (Nozais, 2003). The breakup of Africa and South America has also been used to explain

and support the split between Old and New World species of *Leishmania* (Fernandes et al, 1993) after which varied adaptations to changing environmental conditions led to the constitution of specific species.

It is interesting to note that *L. chagasi*, which is a New World species, transcribed the PGF_{2α} synthase - like gene, albeit at low levels compared to the Old World species. *L. chagasi* belongs to the *L. donovani* species complex which also includes *L. archibaldi*, *L. donovani*, and *L. infantum*. Quantitative comparisons of nuclear DNA fragment patterns to establish molecular evolutionary relationships within the major lineages of *Leishmania* indicate that the New World *L. chagasi* is very closely related to the Old World *L. infantum* and *L. donovani* indicating a recent separation (Beverley et al, 1987). The close relationship between *L. chagasi* and *L. donovani*/*L. infantum* may therefore explain the transcription of this gene in *L. chagasi* New World species. Complete genome sequencing and comparison of the three *Leishmania* species *L. major*, *L. infantum* and *L. braziliensis* has shown that degeneration of existing genes leading to loss of function accounts for about 80% of the species differences in *Leishmania* (Peacock et al, 2007). However, it is not possible to deduce whether this gene is functional or a pseudogene. Amplification of 1983bp amplicon in *L. braziliensis* and subsequent nucleotide sequence analysis using standard bioinformatics tools proved that the amplicon was not a PGF_{2α} synthase gene but a non-specific product of a single primer PCR (data not shown).

T. brucei PGF_{2α} synthase (*TbPGFS*) shares 61% amino acid identity with *LmPGFS* (Kubata et al, 2000). Therefore, in this study, *TbPGFS* was used to generate rabbit polyclonal antibodies for screening for *Leishmania* PGFS. Western blot analysis confirmed the expression of PGFS in Old World species and not in New World species. Despite the absence of PGFS on the western blot in the New World species of *Leishmania*, some of these parasites are known to produce PGF_{2α} (Kabututu et al, 2003). Our data suggest that in these New World species, PGF_{2α} is synthesized via a different pathway not involving PGFS. This suggestion is supported by existence of alternative PGF_{2α} synthesis pathways in other members of trypanosomatids where the synthesis of PGF_{2α} is carried out by two different families of proteins. Thus, in the African trypanosome *T. brucei*, PGF_{2α} synthesis is catalyzed by an aldo keto reductase (Kubata et al, 2000) whereas PGF_{2α} synthesis in the American trypanosome *T. cruzi*, is catalyzed by *T. cruzi* old yellow enzyme (*TcOYE*) which is a flavoprotein (Kubata et al, 2002). This enzyme is also involved in the metabolism of trypanocidal compounds in the parasite with the generation of free radicals. This was discovered while searching for a PGF_{2α} synthase homologue in *T. cruzi*, when attempts to clone PGF_{2α} synthase gene and purify the protein proved unsuccessful as the gene was a pseudogene (Kubata et al, 2002). Since genomic (RT-PCR) and serologic (Western blot) results of this study demonstrates that PGFS is not expressed in the New world species of *Leishmania*, it is likely that a different enzyme is responsible for PGF_{2α} production representing a system analogous to that seen in trypanosomes.

The vectors for *Leishmania* species are *Phlebotomus* sandflies for the Old World and *Lutzomyia* sandflies in

the New World (Sharma & Singh, 2008). Thus, there exists a selective relationship between each parasite species and its vector based on genetic and ecological factors. For example, attachment of promastigotes to the midgut epithelium of the sand fly vectors which is an essential part of the *Leishmania* life cycle, is partly controlled by species-specific modifications of promastigote lipophosphoglycan (LPG) that selectively binds to the midgut galectin receptor (PpGalec) of the sand fly (Kamhawi et al, 2004, Sacks, 2010). Since PGF_{2α} is more highly expressed in promastigotes than in amastigotes (Kidane et al, 1989) it is likely that selective factors in the sand fly vector could have played a role in the divergence of PGF_{2α} synthase gene between Old and New World *Leishmania*. As this gene is expressed in Old World species and not in the New World species, perhaps this protein is essential in the former, but switched off in the latter for the successful metabolism and survival of the parasite in its vector

Whereas PGF_{2α} is associated with parturition (Xu et al, 2013) and luteolysis (Shirasuna et al, 2012) in mammals, its role in *Leishmania* parasites is not clear. However, studies have shown differential expression of this protein when promastigotes are subjected to drug pressure. In a study that carried out comparative proteomics of Pentavalent antimony (SbV) sensitive and resistant *L. tropica* promastigotes to identify proteins with differential expression, PGF_{2α} synthase was one of 4 proteins that were under expressed in resistant isolates (Hajjarian et al, 2012). In a similar study that carried out proteomic analysis on *L. major* promastigotes to determine the effect of methotrexate (MTX) exposure and resistance on protein expression, PGF_{2α} synthase was one of several proteins that were over-expressed in mutants. Further experiments showed that PGF_{2α} synthase had no direct role in MTX resistance, but was probably required for partial compensation of growth defects caused by that resistance (Drummelsmith et al, 2004). This suggests that PGF_{2α} synthase plays a role in the stress response of the parasite allowing the parasite to survive in the presence of drug pressure.

Studies in the kinetoplastids *Leishmania*, *T. brucei* and *T. cruzi* indicate that prostaglandin production in these parasites is not inhibited by aspirin and indomethacin (Kabututu et al, 2003, Kubata et al, 2000, Kubata et al, 2002). These classical non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit mammalian cyclooxygenase activity thereby preventing the formation of prostanoids (Wilson et al, 2004). Thus, the enzyme pathway for the production of prostaglandins in *Leishmania* is distinct from the mammalian counterpart and may be explored as a drug target for the treatment of *Leishmaniasis*.

This study had one major shortcoming. Whereas the RT-PCR and western blot analyses were able to show expression of PGFS-like proteins in the Old World species of *Leishmania* in the three species, enzyme kinetic studies were not carried out to ascertain that the expressed proteins were indeed the PGF_{2α} synthases. Despite this shortcoming, this study has demonstrated that PGF_{2α} synthase-like proteins are expressed in Old World species of *Leishmania* but not in New World species.

5. Conclusion

Findings of this study have expanded the numbers of Old and New World *Leishmania* species to confirm previous findings that PGF_{2α} synthase is expressed in Old World species and not New World species of *Leishmania* (Kabututu et al, 2003). Functional studies should be carried out to determine the role of PGF_{2α} in the *Leishmania* parasite with a view to explore it as a potential target for novel antileishmania therapy.

Conflict of Interest declaration

The authors declare no conflict of interest

Disclaimer

The views expressed are those of the authors and do not represent the position of United States Department of Defense.

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