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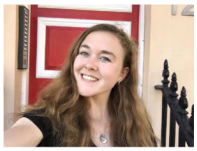
Review Article

Genome plasticity and its role in leishmania adaptation and drug resistance

Rhiannon Moody

College of Medical, Veterinary, and Life Sciences, University of Glasgow,United Kingdom



***Corresponding author:**
Rhiannon Moody ,BSC Hons.
College of Medical,
Veterinary, and Life Sciences,
University of Glasgow,United
Kingdom

rhiannonmoody@mail.com

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ABSTRACT

Objectives: This literature review aims to summarize the current knowledge regarding the genome plasticity observed within the genome of the *Leishmania* parasite, and to discuss how genome plasticity contributes to the adaptation of the parasite and to development of a drug resistant state.

Material and Methods: The search terms "*Leishmania*" and "genome plasticity", were used to search the PubMed database for relevant papers, published between the years 2000 and 2020**.**

Results: Aneuploidy within the *Leishmania* genome allows for drug resistance and adaptation to the environment. In addition copy number variation promotes the up regulation of genes conferring drug resistant capabilities to the parasite.

Conclusion: Drug-resistant *Leishmania* mutants display differential patterns of chromosomal somy when compared to wild-type strains. Highlighting a role for mosaic aneuploidy in the development of drug resistance. *Leishmania* parasites in the amastigote life cycle stage display differential gene copy numbers compared to parasites in the promastigote life cycle stage. Suggesting that copy number variation contributes to parasite adaptation to the environment.

Keywords: Leishmania, Genome plasticity, Leishmania adaptation

INTRODUCTION

Leishmania belongs to the class Kinetoplastea, a grouping of flagellated, protozoan parasites, identifiable by a DNA containing region termed a kinetoplast located within a singular, enlarged mitochondrion.[1] *Leishmania* are responsible for the infectious disease leishmaniasis,[2] transmitted via zoonotic transmission from the phlebotomine sand flies.[3] Infection with different species of the *Leishmania* genus results in different disease states. The species *L.major, L.mexicana, L.amazonensis and L.braziliensis* cause cutaneous Leishmaniasis (CL),[4] whereas the species *L.donovani* and *L.infantum* result in visceral Leishmaniasis (VL).[5] CL is a chronic disease, in which symptoms remain localized to the skin or mucosal surfaces.[4] VL occurs when *Leishmania* disseminates to internal organs.[5] Leishmaniasis is classified as a neglected tropical disease,[3] responsible for approximately 20000–40000 deaths per annum.[6] Leishmaniasis has a wide geographical distribution, with the majority of VL cases concentrated in Brazil, East Africa and India, and the majority of CL cases distributed across the Americas, the Mediterranean basin, the Middle East and Central Asia.[6] Leishmaniasis is a treatable disease with antileishmanial drugs displaying both safety and efficacy. However, the incidence of drug resistant *Leishmania*

infections is increasing, resulting in a corresponding increase in treatment failure.[7] Genetic plasticity within the *Leishmania* genome is thought to promote development of the drug resistant state.[8–10] Furthermore, plasticity of the *Leishmania* genome enables adaptation to parasitic environments, for example the differential gene expression observed within the insect vector and the mammalian host, corresponding to the differing environmental conditions in both hosts, permitting establishment of infection.[8] Such genomic plasticity features include; mosaic aneuploidy, copy number variation (CNV) and genome rearrangements. Understanding the mechanism of genomic plasticity occurring within *Leishmania* allows for the opportunity to target such processes as a means of combating drug resistance. In this essay I will discuss the data evidencing aneuploidy and CNV in *Leishmania,* and the biological implications these plastic features confer to the parasite.

ANEUPLOIDY

Aneuploidy in *Leishmania*

Aneuploidy is defined as the abnormal number of chromosomes within a cell. Aneuploidy is typically detrimental to the fitness of an organism, for example, trisomy of chromosome 21 is the causative agent for Down syndrome.[8] However, microorganisms, including; Candida, *Trypanosoma Cruzi*, and *Leishmania*, have been observed to display aneuploidy.[8,9,11,12] Fluorescence *in situ* hybridization (FISH) and whole genome sequencing (WGS) utilizing Illumina high throughput sequencing followed by Read Depth coverage (RDC) have revealed great variation in chromosome copy number (CCN), both within and among, species, strains, and population of *Leishmania.*[10,13] FISH and WGS demonstrate that the chromosomes of *Leishmania* exist in varying states of ploidy among monosomic, disomic, trisomic and tetrasomic.[13] Such heterogeneity in ploidy expression has been termed as aneuploidy mosaicism.[8-10,13]

FISH indicates that the genome of *L.major* has been shown to be predominantly disomic, with chromosomes 1, 5, and 27 displaying trisomy, and chromosome 2 displaying mainly monosomy.[10] However, CCN did vary among cells of this *L.major* population.[10] Chromosomes 12, 27, and 36, appeared stable in somy expression at a population-level, whereas ploidy in chromosomes 1, 2, 5, and 17, fluctuated between cells of the population.[10] For example; chromosome 1 was observed to be trisomic in 62% of cells and disomic in 37% of cells.[10] As illustrated in Figure 1.

Several methodological strengths increase the validity of the results generated by FISH analysis. To avoid the influence of biases due to artefact in the counting of chromosomes, two color labeling of the same chromosome was performed. Furthermore, all counts were performed blinded and by two

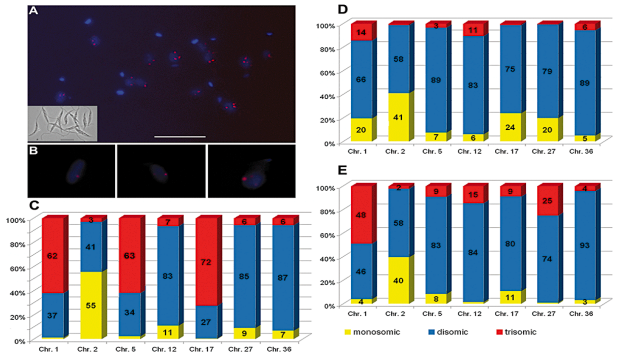

Figure 1: Variable ploidy for seven chromosomes in *L.major* (Amended from Sterkers., et al., 2010).

Figure 1 illustrates aneuploidy and mosaicism in an L.major strain. Numerous chromosomes within the Leishmania *genome display varying levels of aneuploidy, evidencing the high incidence and importance of this genome plasticity feature to the parasite. Variable ploidy expression, in seven chromosomes studied, between cells of an L.major strain. Percentage of mono- di- and tri-somic chromosomes in L.major is demonstrated by the y-axis. Each bar represents one of the seven chromosomes studied. Yellow indicates monosomic, blue indicates disomic, and red indicates trisomic.*

independent counters. The results were additionally verified through the study of two other *L.major* strains obtained from both human and dog. Thus, overall, it appears that FISH evidence for aneuploidy did not result from artifactual genetic drift due to long-term laboratory cultivation. The results generated from this study are in accordance with those derived from a study utilizing a WGS approach to study CCN variation.[13] However, whilst both studies determined the *L.major* genome to be predominantly disomic, the WGS study did not detect high levels of monosomy or trisomy in chromosomes 2, and 1, 5 and 17, respectively. These differences may be accounted for by the differing methodologies. FISH is the only technique capable of discerning the number of aneuploidies within each individual cell in a given population,[10] whilst WGS yields a clearer population wide view of aneuploidy as it analyses many millions of cells.[13] In addition, the WGS study found that, for *L.infantum*, nine chromosomes displayed trisomy. Moreover, in one *L.mexicana* isolate three chromosomes displayed trisomy, whereas in a different *L.mexicana* isolate, only one chromosome was shown to display trisomy, suggesting variation in chromosomes affected. Interestingly, a variable number of chromosomes within *L.mexicana* were of intermediate read-depth, being neither disomic nor trisomic, indicating a mixture of individual cells within the population displaying di- and tri-somy. This phenomenon was also observed for *L.braziliensis*. One conserved finding between all strains and species of *Leishmania* was chromosome

31 always being supernumerary. Results from WGS and RDC, were further confirmed via allele frequency analysis and real time polymerase chain reaction.(RT-PCR) Such reproducibility within the results confers greater reliability to the findings. Nonetheless, there were some exceptions to the general concordance. For instance, chromosomes 5 and 16 in *L.mexicana* were predicted to be disomic by allele frequency analysis, but supernumerary by RDC data. One possible explanation for this discrepancy in data may relate to the decrease in accuracy observed in RDC when detecting multiple copy genes.[13] Alternatively, this discrepancy may be explained as a result of the variable ploidy expressed by cells of the same strains of *Leishmania* species, thus ploidy levels may differ between different isolates of the same strain, resulting in contrasting data when different test isolates are used. Overall, data collated from numerous studies employing different experimental methodologies, evidences the existence of widespread aneuploidy in different Leishmania species.

Aneuploidy as a survival strategy

Biological implications of aneuploidy

Mosaic aneuploidy results in genetic and phenotypic diversity, which may confer a survival advantage to *Leishmania*.[10] Mosaic aneuploidy also permits strain genetic heterogeneity, even in a population of genetically homozygous cells.[14] Displaying polysomy allows for the opportunity to eliminate deleterious mutations, whilst simultaneously retaining beneficial mutations.[15] As in, if a deleterious mutation was present on a monosomic chromosome, then it can be rapidly removed from the population. In line, if a beneficial mutation occurs on a disomic or trisomic chromosome, then expression of this mutation will be greatly upregulated. Aneuploidy allows the parasite to incur the advantages possessed by both haploid and diploid genomes.[15] Mutations occurring within a haploid genome tend to have immediate phenotypic effects. Therefore, in situations of rapidly changing environments, beneficial mutations can be rapidly selected for, promoting survival. Diploid genomes are more successful in terms of mitigating the effects of deleterious mutations, for instance trisomy protects against potentially harmful mutations as it is highly unlikely that the same mutation will be present on all three homologues. Thus, heterozygosity may prevent the expression of particular disadvantageous mutations. A further survival advantage posed by aneuploidy is the alteration of gene expression, which has implications for development of drug resistance and the adaptation to the environment.[17,18,19]

Aneuploidy and drug resistance

Utilizing DNA microarray data, one study demonstrated that ploidy differed between a methotrexate (MTX)-drug-resistant strain of *L.major* and a wild-type strain.[17] The chromosomes 22 and 28 displayed polysomy in the drug resistant strain, whereas the chromosomes 11 and 12 displayed monosomy. Such patterns were not present in the wild-type strain. These findings indicate that drug treatment provides a selective pressure upon *Leishmania* and CCN. Thus, demonstrating the role of ploidy in the development of drug resistance. This finding is strengthened by the presence of a control group (the wild-type strain) in this study, thus enabling comparison, improving the internal validity of the research. Of note, the genes underlying the generated drug resistance on the aforementioned chromosomes have not been identified. Therefore, further genome analysis studies, employing reverse genetics methodologies, are necessitated to elucidate the responsible genes.

Aneuploidy and adaptation to life cycle stage

Differences in CCN have been observed between life cycle stages of *Leishmania*, suggesting that aneuploidy may play a role in *Leishmania* adaptation to host.[8,18,19] The environment of the insect vector differs greatly with respect to the environment of the mammalian vector, in terms of pH, temperature, and nutrients.[18] Thus, the ability to display ploidy variation, and thus, upregulate gene expression through polysomy, and downregulate gene expression through monosomy, the parasite is able to regulate gene expression in response to the environment. Evidencing the ability of aneuploidy to regulate gene expression, correlations have been observed between CCN and corresponding gene transcript levels.[19] Such gene expression will differ between promastigote and amastigote life cycle stages, in concordance with the differing environments the parasite is exposed to.

RNA-sequence analysis of an *L.mexicana* strain demonstrated that within the amastigotes stage, chromosome 30 exhibited polysomy. Importantly chromosome 30 encodes several factors that aid survival within the mammalian vector, including amastins (transmembrane glycoproteins involved in *Leishmania* survival within the mammalian host[21]), and amino acid transporters.[8] Similarly, another study determined that somy differed between promastigote and amastigote life cycle stages.[19] Amastigote adaptation was linked to a decrease in somy of chromosomes 5, 9, 16, 23, and 26.[19] In addition, increases in somy were reported in chromosome 8 and 10, linked to amastigote adaptation.[19] Chromosome 8 encodes amastins, which may explain the pattern of chromosomal somy displayed. Chromosome 10 contains genes encoding the glycoprotein 63 (GP63) which is a protective factor during the amastigote stage of the life cycle, demonstrating that aneuploidy aids in *Leishmania* adaptation to life cycle stage and host environment. Causation is a frequent limitation of the aforementioned studies. Determination of which gene on each chromosome is the causative factor of the survival advantage, has not been sufficiently evidenced.

In summary, aneuploidy within the *Leishmania* genome allows for drug resistance and adaptation to the environment, as exemplified from data derived from a multitude of studies.

Mechanism of aneuploidy generation

Mosaic aneuploidy, as observed in *Leishmania,* is thought to result from a lack of control regarding chromosomal replication, thus resulting in over or under replication of the chromosome, and, consequently, gain or loss of the chromosome copy, respectively.[10] Post-translationally modified histones may modify auto replicating sequences via epigenetic mechanisms, resulting in the defective chromosome replication control.[15] Alternatively, mosaic aneuploidy may arise following chromosome mis-segregation.[7] Asymmetric segregation of chromosomes can be observed during mitosis, with dividing cells displaying asymmetric allotment of chromosome homologues into the two daughter cells in either a 1+2 or a 2+3 pattern.[14] Such that the total number of chromosomes is always odd.

GENE MODIFICATIONS

Gene copy number variants

Leishmania is possessive of the ability to both amplify and delete specific DNA sequences.[22] One mechanism by which *Leishmania* is able to modify gene expression is through induction of gene CNVs.[8] DNA amplification can result from homologous recombination (HR).[23,24] DNA amplifications can be observed as the formation of extrachromosomal regions of the *Leishmania* genome, as either circular elements or linear minichromosomes, formed through rearrangements of direct or inverted homologous repeated sequences, respectively.[22,17] Alternatively, HR can promote intrachromosomal tandem duplication.[22] Variation in gene copy number frequently occurs in response to parasite environment and selection pressure, such as that exerted by drug administration.[22,17]

Gene copy number variation and drug resistance

Genome plasticity, in the form of variations in gene copy number, has been implicated in the development of drug resistance, as evidenced by significant differences in gene expression between MTX-drug-resistant *L.major* and *L.infantum* species and wild-type strains,[17] as demonstrated in Figure 2.

Utilization of a comparative wild-type group in the study, affords greater validity to this conclusion. Gel electrophoresis data exemplified gene amplification in the *DHFR-TS* gene, present on chromosome 6, in MTX-drug-resistant mutants.[17] This finding was further confirmed via PCR.[17] Such reproducibility of these findings improves the reliability of the data. *DHFR-TS* was amplified on circular extrachromosomal

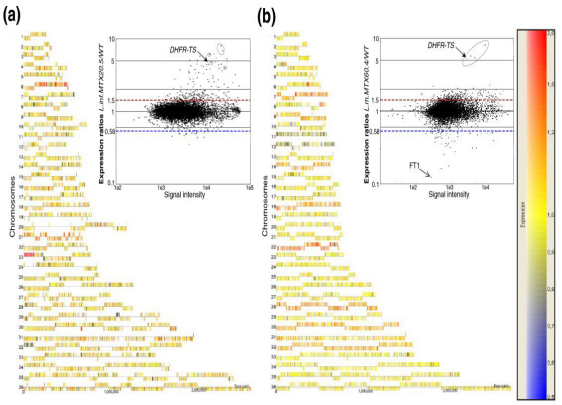

Figure 2: DNA microarray data highlighting differential gene expression in MTX-resistant and wild-type *L.infantum* [amended from Ubeda et al., 2008].

The figure illustrates numerous genes which are differently expressed between the wild-type L.infantum *and the MTX-resistant* L.infantum, *indicating the possibility that copy number variation, and consequent up- or down- regulation of genes, contributes to the development of MTX resistance. The red line on the scatter plot indicates a 1.5 fold increase in gene transcription, with the blue line indicating a 1.5 fold decrease in gene transcription, between the wild-type* L.infantum *and the MTX-resistant* L.infantum, *the y-axis represents the expression ratio between mutant and wild type cells, and the x-axis represents signal intensity. One of the genes demonstrating a 1.5 fold increase in expression between MTX-resistant* L.infantum *and wild-type* L.infantum, *was identified as* DHFR-TS.

DNA regions through the process of HR between repeated sequences. *DHFR-TS* upregulation results in increased production of DHFR, a key enzyme in parasite DNA biosynthesis.[25] The same study also reported linear amplification of the *PTRI* gene in MTX-resistant mutants.[17] The *PTRI* gene encodes the protein PTRI, whose enzymatic activity is similar to that of DHFR, and thus, is an important determinant to parasite survival.[26] Both the *DHFR-TS* and the *PTRI* genes are flanked by numerous repeated sequences, thus providing a mechanism from rapid amplification of these metabolic genes in situations of limited nutrients. DNA deletion events can also promote drug resistance. For example, the *FT1* gene, which encodes folate transporters, is downregulated in MTX-resistant *L.major* and *L.infantum* mutants.[17] MTX gains entry to cells via folate transporters, thus *FT1* downregulation results in decreased uptake of MTX.[27] Similarly, cosmid-sequencing demonstrated a significant difference in gene CNVs between wild-type and MTX-drug-resistant *Leishmania* strains.[22] One limitation of this methodology, however, is the inability to detect loss-of-function mutations, and therefore, cannot be utilized to confirm the finding of *FT1* downregulation. Furthermore, linear amplification has also been shown for the ABC transporter encoding gene *MRPA,* in *L.major* and *L.infantum*

strains resistant to antimonials.[17] Amplifications promoting drug resistance confer a survival advantage to the parasite, and thus, are positively selected for. Illustrated by increased presence of amplifications in *L. major* and *L.infantum* isolates exposed to drugs, when compared to unstressed controls.[17] Contrastingly to gene amplifications occurring within extrachromosomal regions, gene amplification can also occur in intrachromosomal regions of the *Leishmania* genome and can confer drug resistance to the parasite.[28] Next generation sequencing (NGS) revealed DNA amplification on chromosome 19 to occur on intrachromosomal, as opposed to extrachromosomal, regions.[28] This finding was further validated by RT- PCR.[27] Similar to previous studies, NGS demonstrated that gene deletions also result in drug resistance.[25] DNA deletion on chromosome 31 in the aquaporin 1 (AQP1) encoding region has been linked to *Leishmania* resistance to antimony. The association between *AQP1* downregulation and *Leishmania* antimony resistance has been found in both laboratory cultured *Leishmania* strains and field isolates, conferring greater external validity to this finding. AQP1 provides a route of entry for antimony compounds in *Leishmania*, thus providing an explanation for the resistance acquired in the absence of this transporter protein.

Overall, evidence drawn from multiple studies demonstrates a role for CNV in the upregulation of genes conferring drug resistant capabilities to the parasite.

Gene copy number variation and adaptation to environment

Numerous studies have reported gene CNVs in the DNA regions encoding proteins, including; amastins, GP63, and peptidases.[8,29,30] Genomic amplification in these encoding regions, and the consequent upregulation of the encoded proteins, promotes parasite survival within the host. Amastins are cell surface expressed proteins that aid in parasite adhesion to host cells.[8,29] The amastin repertoire has been reported to vary between *Leishmania* species, which may reflect parasitic adaptation to the variety of host environments each *Leishmania* species infects.[8] GP63 is another parasite protein involved in cell adhesion. Within the sand fly host, GP63 functions in midgut attachment. Whereas within the vertebrate host, upregulation of this protein is associated with inhibition of the complement pathway via complement mediated cleavage of C3, and inhibition of antiparasitic activity via interaction with JAK kinases.[8] Similar to the amastin protein family, GP63 expression also varies between *Leishmania* species, with limited activity of GP63 reported for *L.tarentolae* in comparison with the *L.vianna* species, which is reflected by the lack of intracellular life cycles stages for *L.tarentolae* and the different hosts each species infects. Peptidase expression prevents parasite clearance, by down regulating the host T-helper-1-cell immune response.[8] One common methodological limitation present in studies investigating gene CNV, is the focus upon *Leishmania* strains from a specific, singular geographical location, as variation may occur both inter- and intra-species based upon geographical location. Therefore, studies focusing on a singular geographic location may lack both external validity and generalizability. The amplification of differing genetic regions promoting parasite continual survival between *Leishmania* species and intra-species life cycle stages, suggests gene amplification acts as an adaptive survival strategy to differing host environments.

CONTROL OF *LEISHMANIA* DNA REPLICATION

Leishmania transcriptional control

Leishmania parasites exert unique transcriptional programs. For the majority of eukaryotes, each protein is encoded by a single transcription unit, with its own promoter and terminator. In contrast, kinetoplastid, including *Leishmania*, genes are transcribed collectively as part of a polycistronic transcription unit (PTU), and each PTU has the ability to transcribe up to hundreds of genes.[31] *Leishmania* possesses unique DNA replication initiation pathways, as the origin of replication sites are detected in a single region within each chromosome, as evidenced by marker-frequency-analysis.[35] This is in direct contrast to eukaryotic genomes, in which multiple origins of replication sites are located across the linear chromosome.[35] Whilst a multitude of studies evidence unique DNA replication programs existing within the *Leishmania* genome, evidence is currently lacking to explain the fitness advantage posed by the possession of such unique replication mechanisms. The ability to exert a unique mechanism of DNA replication and control, and the resultant genomic plasticity, may provide the parasite with the ability to modulate its genome to respond to environmental stresses, such as the selection pressure exerted via drug administration. Therefore, favoring parasite resistance, consequent survival, and establishment of infection. However, studies evidencing this hypothesis are required, for instance studies comparing parasite response to drug pressure between *Leishmania* species expressing such unique DNA replication pathways, and biologically similar parasite species who lack such unique DNA replication pathways.

Homologous recombination in *Leishmania*

HR is a mechanism whose activity ensures completion of DNA replication. In addition, HR also functions in DNA repair.[31] HR is regulated by the enzyme Rad51 recombinase. HR factors such as Rad51 have been proven to function in episome formation, which in turn has been linked to the

acquisition of drug resistance.[31] MER11 is another factor proven essential in the process of DNA repair, MER11 action is essential to the functionality of Rad51[35,36,37]. MRE11 is responsible for resection of the ends of DNA double-strand breaks.[31] Similar to Rad51, MRE11 promotes the formation of episomes, specifically extrachromosomal linear amplicons, in response to drug pressure.[31] In contrast, Rad51 favors circular amplicon formation.[31] Linear amplification of the *PTR1* gene, mediated by MRE11, and circular amplification of the *DHFR-TS* gene, mediated by Rad51, has been shown to lead to MTX resistance in *Leishmania*.[31] The majority of studies investigating HR in *Leishmania* utilize a common experimental model.[23,31-40] Such standardization in methodology imparts greater reproducibility and reliability to the results generated from such studies. Consequently, the conclusions are afforded greater internal validity.

CONCLUSION

Leishmania is the causative agent of the potentially fatal, infectious disease, Leishmaniasis. With the increasing emergence of drug resistance and treatment failure, the identification of factors contributing to drug resistance is critical. Genome plasticity is a mechanism which can confer drug resistance and adaptation capabilities to the *Leishmania* parasites.

FISH and WGS studies evidence heterogeneity in ploidy expression within the *Leishmania genome.* Differential ploidy expression has been observed between drug-resistant *Leishmania* mutants and wild-type strains. Highlighting a role for mosaic aneuploidy in the development of drug resistance, as a result of upregulation of drug resistant mutations occurring on multiple copy chromosomes. Furthermore, variation in gene copy number is also able to confer drug distance via the amplification of genes encoding drug resistant factors, and the downregulation of genes encoding transport proteins targeted by antileishmanial drugs.

In addition, differential copy number variation has been reported between the amastigote and promastigote life cycle stages of the *Leishmania* parasite. Suggesting that copy number variation contributes to parasite adaptation to the environment.

Understanding the mechanisms responsible for the generation of drug resistance in *Leishmania* parasites enables the development of antileishmanial treatments which either circumnavigate or directly target these mechanisms. Thus, promoting treatment success. Additionally, understanding the mechanisms allowing *Leishmania* adaptation, allows for the development of treatments targeting these mechanisms. The present review demonstrates the functional implications resulting from variation in gene expression. Therefore, a personalized medicine approach may aid in the treatment of *Leishmania* infections. The sequenced *Leishmania* genome and subsequent identification of differential gene expression may allow prediction of treatment failure and has the potential to direct the choice of drug. Furthermore, the identification of differential gene expression between the promastigote and amastigote forms of the *Leishmania* parasites may aid in the development of therapeutic strategies which are tailored to the life cycle stage.

A limitation of the current literature is a lack of causality associated with studies. Consequently, further research utilizing reverse genetic analytic studies is required to elucidate and determine the causal genes responsible for drug resistant states. Furthermore, forward genetic studies are necessitated to determine the phenotypic effect of aneuploidy and CNV in specified chromosomes and genes of the *Leishmania* genome.

Declaration of patient consent

Patient's consent not required as there are no patients in this study.

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Conflicts of interest

There are no conflicts of interest.

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