Drug-Resistance Biomarkers in *Leishmania infantum* through Nanopore-Based Detection of Aneuploidy and Gene Copy Number Variations with LeishGenApp

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Abstract

The emergence of drug-resistant strains of *Leishmania infantum,* a parasite that infects dogs and humans, poses an increasing threat, underscoring the need for a One Health framework for adequate control. Aneuploidy and gene copy-number variation (CNV) are associated with resistance to first-line drugs. We analyzed aneuploidies, genome plasticity, and 15 CNVs as drug-resistance biomarkers in 35 *L. infantum* isolates from human and canine host across the Mediterranean by nanopore-only whole-genome sequencing and the automated diagnostic platform LeishGenApp. Results highlighted different levels of aneuploidy turnover among chromosomes and characterized the structural organization of resistance-associated genomic regions, emphasizing the role of genomic repeated sequences in the extended H-locus, METK and SMT locus. At least one genomic resistance biomarker was detected in 80% of the Mediterranean isolates, being the most prevalent to antimonials (50%) and allopurinol (50%) in canine isolates and to antimonials (61%) and paramomycin (38%) in human isolates. Monoresistance (50%) predominates in dogs, while resistance to two drugs is most common in human isolates (38%). Differences in genomic resistance biomarkers prevalence in our cohort underscore the importance of targeted drug policies to mitigate resistance dissemination. Our results provide an effective approach for detecting aneuploidies and CNVs as drug-resistance biomarkers in *Leishmania* parasites by nanopore-only whole-genome sequencing. This powerful tool is significant for monitoring resistant *L. infantum* strains, particularly in dogs, the primary

reservoir, lessening resistance-related relapses, limiting resistant *Leishmania* transmission by sand flies, and shaping One Health strategies.

Introduction

Leishmania (L.) infantum is the only autochthonous *Leishmania* species in the Mediterranean and peri-Mediterranean area, causing both canine (CanL) and human zoonotic visceral leishmaniosis (HumL), with two main clinical forms: visceral (VL) and cutaneous (CL) leishmaniosis. In these areas, where the disease is considered endemic, CanL affects approximately 2.5 million dogs annually (Moreno & Alvar, 2002) and over a 15-year period (2005-2020), WHO reported 8.367 cases of HumL (Maia et al., 2023) by *L. infantum*. The notification of CanL and HumL cases to the World Organization for Animal Health (WOAH) and the World Health Organization (WHO), respectively, is mandatory in all these endemic countries, except France (ECDC, 2022).

The standard treatment for CanL combines leishmanicidal drugs, such as meglumine antimoniate or miltefosine (approved in Europe in 2007), with the off-label leishmaniostatic drug allopurinol, administered for long-term (6-12 months). For HumL in the Mediterranean region, liposomal amphotericin B (first registered in 1996 and exclusive to HumL use) is now the treatment of choice, replacing pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) as the first-line drug, which had been in use since the 1920s (Gradoni et al., 2017). Other alternatives to pentavalent antimonials now include miltefosine (first registered in India in 2002 (Pérez-Victoria et al., 2006) but with efficacy not proven in the treatment of European VL) and paromomycin (incorporated in 2006) (Matos et al., 2020).

Leishmania treatment faces a critical hurdle with the increasing rate of emerging strains resistant to first-line drugs (Leprohon et al., 2015). Drug Resistance (DR) is a significant contributor to therapeutic failure (Ponte-Sucre et al., 2017) and renders current antileishmanial drugs less effective, requiring dose increase or prolonging the treatment time, with the associated side effects (*e.g.,* nephrotoxicity). Resistant strains of *Leishmania* have been identified in infected dogs after treatment (Gramiccia et al., 1992; Yasur-Landau et al., 2016; Gonçalves et al., 2021,Schäfer et al., 2024) as well in humans (Cojean et al., 2012), and their clinical-derived *Leishmania* promastigotes were studied *in vitro*. Moreover, differences in drug susceptibility among *Leishmania* strains depending on their geographical location complicate treatment guidelines standardization (Berman et al., 1998; Musa et al., 2010). To address these issues, combined drug therapies, especially in dogs, are increasingly recognized as a strategy to delay the emergence of resistance, although risks remain (Leprohon et al., 2015). Unhopefully, alternatives are scarce with few new drugs in the pipeline, and the absence of effective vaccines.

The genome of *L. infantum* (32 Mb; 36 chromosomes) exhibits significant plasticity, characterized by aneuploidy and gene copy number variation (CNV), both hallmarks of the genus and known mechanisms of gene dosage control, in the absence of classical transcriptional regulation (De Gaudenzi et al., 2011). These features occur naturally, are well tolerated by the parasites, and are enhanced in response to stress (*e.g.,* drug pressure) (Prieto Barja et al., 2017). Increased dosage in *dihydrofolate reductase thymidylate synthase* gene (*dhfr-ts)* was the first example of CNV through gene amplification in methotrexate-resistant *Leishmania* (Coderre et al., 1983). Similarly, the *multidrug-resistance protein A* gene (*mrpa*) amplification into circular (Leprohon et al., 2009) or linear minichromosomes (Ubeda et al., 2014), is associated with trivalent antimonials resistance, the active form resulting of the pentavalent antimonial prodrug reduction. Other examples include reducing flippase activity through *L. donovani miltefosine transporter* gene

(*LdMT)* deletion, which diminishes miltefosine accumulation (Pérez-Victoria et al., 2003). Multicopy tandem gene changes can crucially affect drug sensitivity, as seen with the *metk* genes and their response to allopurinol (Yasur-Landau et al., 2018). These examples reinforced *Leishmania*'s genomic features in the diverse adopted mechanisms generating genome variability in response to stressors. Other biomarkers previously reported associated with resistance are described in more detail in Table 1.

Given the challenge of increasing drug resistance in *L. infantum* strains, we analyze its prevalence in a cohort of isolates from an endemic region as the Mediterranean basin by means of LeishGenApp, an innovative genomic platform that leverages nanopore long reads and the automated analysis of a panel of 15 drug-resistance biomarkers to the most common treatments for clinical CanL and HumL. As reported in a previous study (Martí-Carreras et al., 2022), long reads obtained by nanopore sequencing platforms (Oxford Nanopore Technologies, plc) resulted in near-complete *Leishmania* genome assemblies, the full characterization of large structural variants (>1 kb), and the obtention of a full spectrum of structural variants and mutations. After whole-genome sequencing 35 *L. infantum* isolates, LeishGenApp offers a comprehensive analysis of both identified and candidate drug resistance drivers, outperforming traditional targeted molecular methods, and provides relevant information that optimizes treatment selection for CanL and HumL.

Material and Methods

Samples

Sixty *L. infantum* samples from promastigote cultures with parasite loads ranging from 10^6-10^8 parasites/mL or *Leishmania* DNA samples were gathered from reference promastigotes collections or grown from collected clinical samples. Additionally, ATCC Control DNA was acquired from reference *L. infantum* strain Nicolle (ATCC 50134D, MHOM/TN/80/IPT-1). Of the 60 *L. infantum* samples, 48 isolates were whole genome sequenced, obtaining 35 isolates above the described quality threshold for CNV analysis (see below). The final 35 samples of this study cohort came from different origins: Italy (Sicily (15), Spain (13), Portugal (4), Israel (2), and Tunis (1; the ATCC MHOM/TN/80/IPT-1 Control DNA). Of these, 22 samples were isolated from dogs and 13 from humans (Table 2).

The cultured samples were propagated in different media depending on their institution of origin: M199 medium, NNN medium cryopreserved in RMPI medium with 20% fetal bovine serum (FBS) and 10% DMSO, Evans' modified Tobie's medium, or Schneider medium (S0146-500ML, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% FBS 1% human urine, and 25 mg/mL gentamicin, corresponding to cultures grown in Israel, Portugal, Sicily or Spain, respectively.

DNA extraction and Nanopore Sequencing

DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (D4300; Zymo Research Corporation; Los Angeles, CA, USA). DNA quality and quantity were determined using a NanoDrop 2000 Spectrophotometer and a Qubit dsDNA HS Assay Kit (Fisher Scientific). Nanopore sequencing libraries with up to 2 barcoded samples per sequencing run were prepared with 200 ng of genomic DNA using the Rapid Barcoding Sequencing Kit (SQK-RBK004; Oxford

Nanopore Technologies, ONT) and loaded in a MinION FLO-MIN106 v9.4.1 flowcell or with 1000 ng for the Native Barcoding Kit 24 V14 (SQK-NBD114.24; Oxford Nanopore Technologies, ONT) into a MinION FLO-MIN114 v.10.4.1 flowcell, respectively. Sequencing was performed on a GridION (GridION, ONT, Oxford, UK) for 48h. The fast5 files were basecalled and demultiplexed, and the adapters were trimmed using Guppy (v6.1.2-v7.1.4; "SUP" model) or Dorado (v7.2.13; "SUP" model).

Aneuploidy and gene copy number variation analysis

Aneuploidy and gene copy number variation (CNV) were determined using the LeishGenApp v.2.2.0 analysis platform (Nano1Health S.L., Barcelona, Spain), as previously described (Martí-Carreras et al., 2022). Briefly, uncorrected nanopore reads were mapped against *L. infantum* reference (*L. infantum* JPCM5 v2/2018, http://leish-esp.cbm.uam.es/l_infantum_downloads.html, accessed during 1 April 2022) (González-De La Fuente et al., 2017) using LRA v1.3.7.2 (Ren & Chaisson, 2021). The read alignments were processed by SAMTools v1.19.2 (Li et al., 2009). Copy number of the 36 chromosomes and gene CNVs were obtained from read depth coverage in non-overlapping genomic windows of 10kb for chromosomes, and 1kb for the gene CNVs using CNVkit v0.9.10.1 (Talevich et al., 2016).

The chromosome copy number was determined by calculating the median of the $\log_2$ ratio of observed vs. expected copies (2N) derived from read depth coverage measurements. The range for monosomy, disomy, trisomy, tetrasomy and pentasomy was defined based on a ploidy threshold of ±0.5, as described in Patino et al., 2019.
Gene copy number was calculated by the logarithmic transformation of the $\log_2$ ratio of observed vs. expected gene copies (2 copies) derived from read depth coverage for the developed panel of 15 drug target and transporter genes. Whole-genome sequenced samples with coverage lower than 10X for chromosomes containing resistance biomarkers genes were excluded from the analysis. A threshold for biomarker detection of ±0.50 from the sensitive-linked copy number (2, 3, or 4 copies) was used, considering a 5% natural polymorphism within the population (Choudhury et al., 2014), and the LeishGenApp-associated variability (±0.38, data not shown).

Genome *de novo* assembly and annotation

Genomes were *de novo* assembled using N1H-Assemblatron v2.1.1 (Nano1Health SL, Barcelona, Spain) discarding reads with Phred-score lower than 10 by Filtlong v0.2.1. Data summary statistics were obtained using Nanoplot v.1.42.0 (De Coster & Rademakers, 2023). Flye v2.9.1-v2.9.3 (Kolmogorov et al., 2019) was used for *de novo* assembly and contigs were polished using Medaka v1.7.2-2.0.0 (https://github.com/nanoporetech/medaka). Genome completeness was assessed using Busco v5.4.45-v5.8.0 (Manni et al., 2021). Genomes were annotated using online Companion platform v2.2.8 (Steinbiss et al., 2016).

Identification of repeated sequences in the drug resistance associated loci of *L. infantum*

Unknown and described repeats were screened within the extended H-locus regions: LinJ.23:59772-106565, the METK locus: LinJ.30:1280005-1294327, and the SMT locus: LinJ.36:952046-960534 in the *L. infantum* reference genome JPCM5 (GenBank accession number GCA_000002875.2). Locations of the repeated sequences were identified with nucmer (-

l 50) (Marçais et al., 2018) and blastn (part of blast+) (Camacho et al., 2009). The repeated sequences observed in the reference genome JPCM5 were identified in the *de novo* assemblies of our 35 isolates using the same alignment tools. IGV v2.16.1 (Thorvaldsdóttir et al., 2013) was used to visualize, analyze, and describe the structure of regions with repeated sequences.

Data analysis and visualization

A linear regression model assessed whether mid-term culturing significantly explains aneuploidy levels. The model included one factor with two levels: clinical isolates (<10 passages, n=10) and culture lines (10–33 passages and/or subjected to a freezing step, n=9). See Table 2 for in detail sample information. Assumptions of homoscedasticity (Bartlett's test: $K^2 = 0.345$, $p = 0.556$) and residual normality (Shapiro-Wilk: $W = 0.967$, $p = 0.229$) were met. Data analysis and visualization were performed using R-packages, including tidyverse (Wickham et al., 2019), and BioCircos (Cui et al., 2016), using R studio interface running R version 4.4.1.

Results

Detection of aneuploidies and genome plasticity in *L. infantum* genome by nanopore-only whole-genome sequencing

Sixty *L. infantum* isolates of canine and human origin from the Mediterranean basin, an endemic region for CanL and HumL, were gathered. The isolates came from Italy (20), Portugal (20), Spain (13), and Israel (7), with titers ranging from 10^6 to 10^8 parasites/ml. Forty-three isolates with titers higher than 10^7 parasites/ml were whole genome sequenced with a N50 ranging between 6-9 kb. After nanopore-only sequencing, 35 high-quality genomes isolated from canine (22) and human (13) samples were assembled, with an average genome coverage of 55X and a completeness of 97%. Eight samples with lower parasite concentrations were discarded because they did not meet the criteria for analysis (genomic sequencing depth of the chromosomes > 10X). Data is available in the DDBJ/ENA/GenBank repository under the BioProject PRJNA881045 with the following accession numbers: JAZFNU000000000-JAZFNZ000000000; JAZFOA000000000-JAZFOZ000000000 and JAZFPA000000000-JAZFPC000000000.

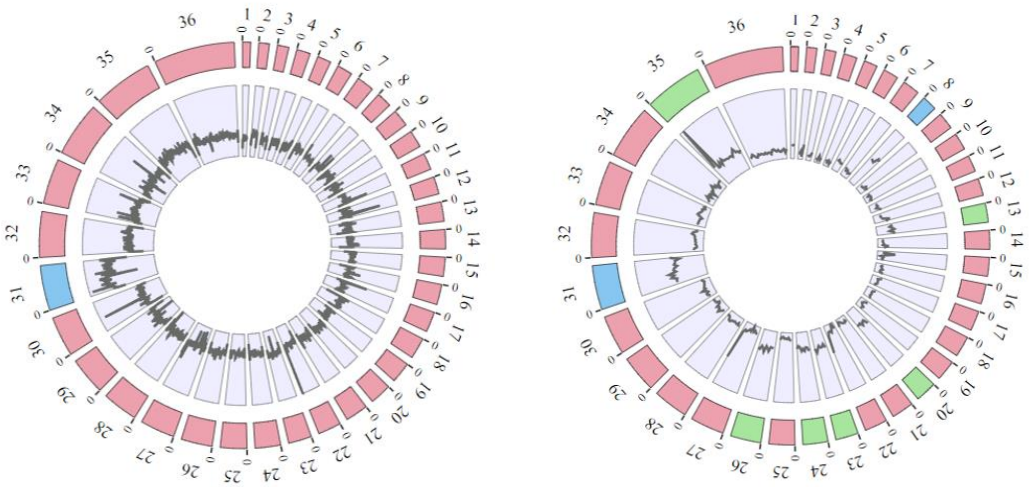
The detailed analysis of the assembled *L. infantum* genomes reveals significant structural variability at both the chromosomal and sub-chromosomal levels. Read-depth analysis shows isolate-specific variations in some of the 36 chromosomes, with either increases or decreases in coverage in the whole chromosome. Notably, all genomes showed a consistent two-fold increase in coverage of chromosome 31. The even distribution of read depth along the whole chromosome 31 suggests a complete whole-chromosome duplication rather than local amplification of specific areas of the chromosome (Figure 1a). Further analysis revealed that chromosomes can be assigned to three groups considering their somy: chromosomes 7, 14, 18, 19, 27, 28, 34, and 36 with disomy in at least 95% of the cultures; chromosome 31 was dominantly tetrasomic (88% of samples; sd = 0.29) as expected for *L. infantum* (Rogers et al., 2011); and the 27 remaining chromosomes with variable somy between cultures. Our approach revealed that trisomy is commonly observed in chromosomes 8, 20, and 23 (Figure 1b), although chromosomes 8 and 23 exhibited high variability (sd ≥0.87). Chromosome 23 mean copy number was 2.80, while the median was 2.48, suggesting the presence of extreme somy values in certain isolates, consistent with the high standard deviation. It is noteworthy the widespread polyploidy in the isolates analyzed, with 12 samples (33%) having at least ten chromosomes with somy levels above two. On the contrary,

monosomy was unusual with only two distinct cultures showing monosomy for specific chromosomes (MHOM/ES/2016/CATB101: chr. 27, and chr. 34; LCAN/ES/2023/CATB109: chr. 1). Only one clinical isolate (LCAN/ES/2024/CATB110) had the expected disomic background for all the chromosomes, except for the expected tetrasomic chromosome 31.

When comparing clinical isolates (<10 passages, n=10) and culture lines (10–33 passages, n=9) (see Table 2) for aneuploidy level, no significant differences explained by mid-term culturing were detected ($F = 2.383$, $R^2 = 0.122$, $p = 0.141$). The low R^2 suggests the influence of other factors or a non-linear relation between culturing and aneuploidy. Control DNA from reference *L. infantum* IPT-1 and sample MCAN/IT/2020/1265 (68 passages) had the highest number of aneuploidies (10 and 11 chromosomes beyond 31, respectively) and were excluded from the analysis as they were considered clonal lines due to their high number of passages.

Moreover, as shown in Figure 1b, chromosomes do not have an integer number but intermediate somy values, a phenomenon named mosaic aneuploidy. Notably, the aneuploidies identified in chromosomes 23 and 31 are of particular interest, as these chromosomes harbor 7 of the 15 drug-resistance biomarkers under investigation (see below).

a)



LCAN/ES/2024/CATB110 LCAN/ES/2023/CATB108

b)

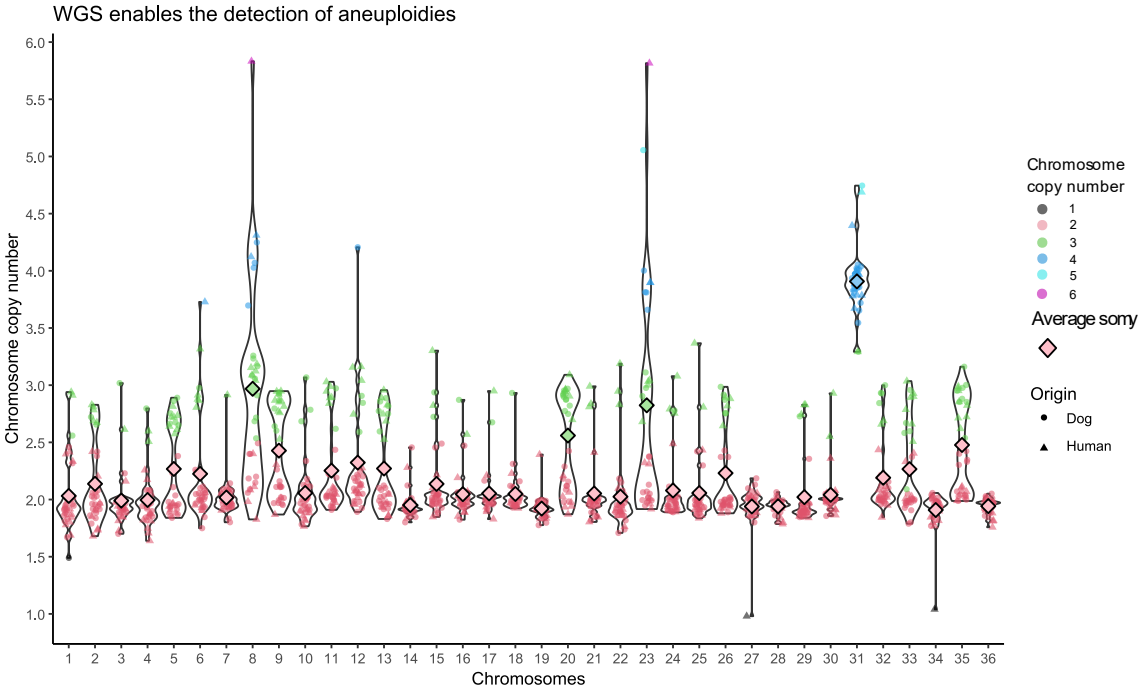


Figure 1. WGS enables the detection of aneuploidies and mosaicism. a) Circular representation of whole-genome coverage by BioCircos. The outer circle represents the 36 chromosomes, and the inner circle represents the coverage along each chromosome. Sample LCAN/ES/2024/CATB110has the expected disomic background except for tetrasomic chromosome 31. Sample LCAN/ES/2023/CATB108 is aneuploid for chromosomes 13, 20, 23, 24, 26 and 35 (trisomy) and 8 and 31 (tetrasomy). b) Violin plots showing the chromosome copy number for each of the 36 *L. infantum* chromosomes across all samples. The median somy for each chromosome, calculated as the median copy number across the 35-sample cohort, is shown by the bigger rhombus.

Copy number variation as drug resistance biomarkers in *L. infantum*

To detect drug-resistance biomarkers in the isolates analyzed, fifteen key drug target and transporter genes were selected, corresponding to eight loci, from *L. infantum*: METK, LdSMT, AQP1, LdMT, LdRos3, MSL, PPM, and extended H-locus (38kb region containing the *abcc1*and *abcc2* transporters genes plus the classic H-locus genes*, mrpa* and *ptr1*). These loci have been previously associated with resistance to the five most common drugs used in the treatment of leishmaniosis in both veterinary and human medicine: allopurinol, amphotericin B, pentavalent antimonials, miltefosine, and paromomycin (see Table 1).

Table 1. 15 drug target and transporter genes associated with resistance to the five first-line drugs in *L. infantum* treatment. Summary of resistance, biomarker, gene ID, gene name, function, and reported gene copy number variation (CNV) as resistance biomarker of the eight loci analyzed with LeishGenApp. R: Resistant; TF: Treatment failure. References are provided for studies showing threshold resistance effect by CNVs.

Resistance	Biomarker	Gene ID	Gene name	Function	Gene Copy Number Variation	References
Miltefosine R	Miltefosine transporter LdMT	LinJ.13.1590	*LdMT*	Phospholipid transport	Deletion (CN -1, -2)	(Pérez-Victoria et al., 2003) (Pérez-Victoria et al., 2006)
	LdRos3 (associated gene)	LinJ.32.1040	*LdRos3*	Phospholipid transport Vps23 core domain		
Miltefosine TF	Miltefosine sensitivity locus (MSL)	LinJ.31.2370	*LinJ.31.2370*	3'- nuclease	Deletion (CN -2)	(Carnielli et al., 2018)
		LinJ.31.2380	*LinJ.31.2380*	3'- nuclease		
Allopurinol R	METK locus	LinJ.30.3560	*metk1*	S-adenosylmethionine synthetase	Deletion (CN -1)	(Yasur-Landau et al., 2018)
		LinJ.30.3580	*metk2*	S-adenosylmethionine synthetase		
Trivalent antimonials R	Extended H-locus	LinJ.23.0230	*abcc1*	ABC-transporter	Expansion (CN+1)	(Douanne et al., 2020)
		LinJ.23.0240	*abcc2*	ABC-transporter		(El Fadili et al., 2005)
		LinJ.23.0290	*mrpa*	ABC-thiol transporter		(Dumetz et al., 2018)
		LinJ.23.0310	*ptr1*	Pteridine reductase 1		(Negreira et al., 2023)
	AQP1	LinJ.31.0030	*aqp1*	Aquaglyceroporin 1	Deletion (CN -1)	(Gourbal et al., 2004) (Andrade et al., 2016)
Amphotericin R and Trivalent antimonials R	LdSMT locus	LinJ.36.2510	*smt1*	Sterol 24-c-methyltransferase	Deletion (CN-1)	(Rastrojo et al., 2018)
		LinJ.36.2520	*smt2*	Sterol 24-c-methyltransferase		
Paromomycin R	Paromomycin (PMM) resistant locus	LinJ.27.1940	*d-ldh*	D-lactate dehydrogenase	Expansion (CN +1)	(Rastrojo et al., 2018)
		LinJ.27.1950	*b-cat*	Branched-chain amino acid aminotransferase		

Prevalence of drug-resistance biomarkers in *L. infantum* genome

Presence of CNVs was analyzed for the 15 selected biomarkers in the 35 high-quality assembled genomes of *L. infantum* through LeishGenApp, a genomic analysis platform through nanopore sequencing and subsequent bioinformatic analysis developed by Martí-Carreras et al., (2022). The sequencing reads obtained for each isolate are mapped against the reference genome of *L. infantum* (*Leishmania infantum* JPCM5 ASM287v2, PRJEB20254), and the gene copy numbers are determined for each of the selected biomarkers. No differences in CNV detection were observed between sequencing kits (Linear Mixed Model, t = 0.556, p-value = 0.579).

Significant variability was detected in the copy number of the biomarkers associated with drug resistance in the 35 *L. infantum* strains analyzed, with 80% of the isolates (28/35) showing at least one genomic resistance biomarker (Table 2).

The prevalence of resistance biomarkers was different among the five drugs, with higher prevalence for antimonials (54%) and allopurinol (37%), followed by paromomycin (23%), amphotericin B (11%), and miltefosine (9%). Prevalences were consistent in cultures isolated from canine and human samples for antimonials (canine, 50%; human, 61%), and miltefosine (canine, 9%; human, 8%). However, a higher prevalence of resistance biomarkers to allopurinol was present in canine-derived cultures (50%) compared to human isolates (15%), and to paromomycin and amphotericin in human-derived cultures (paromomycin, 38%; amphotericin B, 15%) compared to canine isolates (paromomycin, 14%; amphotericin B, 9%). Genomic resistance biomarkers to antimonials and allopurinol were most prevalent among the canine isolates, while in human isolates resistance to antimonials was most common, followed by resistance to paromomycin (Figure 2a). Regarding the genetic profile, monoresistance predominates in dog isolates (50%), while resistance to two drugs is most common in human isolates (38%), as shown in Figure 2b.

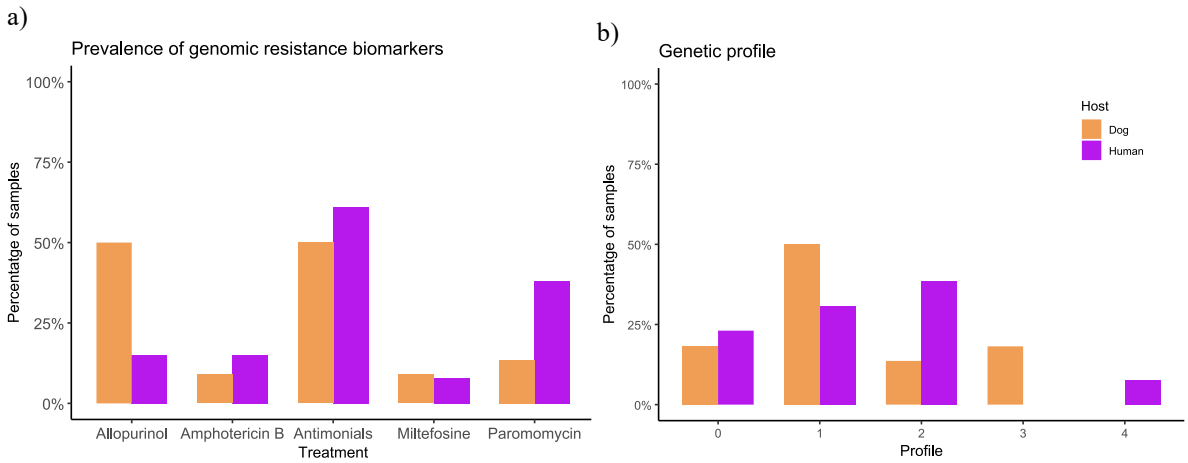


Figure 2. Prevalence of drug-resistance biomarkers in *L. infantum*. a) Percentage of drug-resistance biomarkers detected for each studied drug (allopurinol, amphotericin B, antimonials, miltefosine and paromomycin). b) Percentage of samples with each genomic resistance profile (no resistant, and resistant to one, two, three, and four drugs, respectively). Orange: canine isolated cultures. Purple: human isolated cultures.

Table 2. Sample ID and metadata used in this study. Thirty-five *L. infantum* samples were used in this study. Columns represent the relation with their original host, collection year, geographical location and detected resistance biomarkers by using LeishGenApp.

					Resistance biomarkers				
Sample ID	**Host species**	**Collection year**	**Location**	**Passages**	**Allopurinol**	**Amphotericin B**	**Antimonials**	**Miltefosine**	**Paromomycin**
MCAN/PT/2012/IMT397	*Canis lupus familiaris*	2012	Portugal (Setúbal)	-	METK	-	-	-	-
MHOM/MA/67/ITMAP-263	*Homo sapiens sapiens*	2023	Portugal	-	METK	-	*aqp1*	-	-
MHOM/MA/67/ITMAP-264	*Homo sapiens sapiens*	2023	Portugal	-	-	*smt2*	*abcc1, abcc2, mrpa*	-	-
MHOM/MA/67/ITMAP-265	*Homo sapiens sapiens*	2023	Portugal	-	-	-	*abcc1, abcc2, mrpa, aqp1*	-	-
LCAN/ES/1992/CATB011	*Canis lupus familiaris*	1992	Spain (Catalonia)	-	-	-	*abcc1, abcc2, mrpa, ptr1, aqp1*	-	-
MCRI/ES/2006/CATB033	*Canis lupus familiaris*	2006	Spain (Catalonia)	-	-	-	-	*LdMT*	-
MHOM/ES/2016/CATB101	*Homo sapiens sapiens*	2016	Spain (Mallorca)	11	-	-	*aqp1*	-	*b-cat*
LCAN/ES/2020/CATB102	*Canis lupus familiaris*	2020	Spain (Zaragoza)	8	-	-	-	-	*b-cat, d-ldh*
LCAN/ES/2020/CATB103	*Canis lupus familiaris*	2020	Spain (Zaragoza)	7	-	-	*aqp1*	-	-
LCAN/ES/2022/CATB104	*Canis lupus familiaris*	2022	Spain (Valencia)	4	-	-	-	-	-
LCAN/ES/2023/CATB105	*Canis lupus familiaris*	2023	Spain (Barcelona)	5	-	-	-	-	-
LCAN/ES/2023/CATB108	*Canis lupus familiaris*	2023	Spain (Barcelona)	11	-	-	-	-	-
LCAN/ES/2023/CATB109	*Canis lupus familiaris*	2023	Spain (Barcelona)	15	-	-	-	-	-
LCAN/ES/2024/CATB110	*Canis lupus familiaris*	2024	Spain (Valencia)	5	-	-	*aqp1*	-	-
MCAN/IL/2011/NT2	*Canis lupus familiaris*	2011	Israel	-	METK	*smt1*	*abcc1, mrpa, ptr1, aqp1*	-	*b-cat*
MHOM/TN/80/IPT-1	*Homo sapiens sapiens*	1980	Tunisia (Monastir)	-	METK	*smt1, smt2*	*abcc1, abcc2, mrpa, ptr1*	-	*d-ldh*
MHOM/ES/2022/LLM-2503	*Homo sapiens sapiens*	2022	Spain (Zaragoza)	-	-	-	-	-	-
MCAN/IT/2011/8283	*Canis lupus familiaris*	2011	Italy (Sicily, Palermo)	11	METK	-	-	-	-
MCAN/IT/2022/21SCIACCA	*Canis lupus familiaris*	2022	Italy (Sicily, Agrigento)	6	METK	-	*abcc1, abcc2, mrpa, ptr1, aqp1*	-	-
MHOM/IT/2017/1533U	*Homo sapiens sapiens*	2017	Italy (Sicily, Agrigento)	6	-	-	*aqp1*	-	*b-cat, d-ldh*
MCAN/IT/2018/4586	*Canis lupus familiaris*	2018	Italy (Sicily, Palermo)	20	-	-	*aqp1*	-	-
MCAN/IT/2018/1380	*Canis lupus familiaris*	2018	Italy (Sicily, Palermo)	33	METK	-	-	-	-
MHOM/IT/2008/31U	*Homo sapiens sapiens*	2008	Italy (Sicily, Palermo)	7	-	-	-	-	-
MCAN/IT/2020/1265	*Canis lupus familiaris*	2020	Italy (Sicily, Palermo)	-	METK	-	*abcc2, mrpa*	-	-

MCAN/IT/2022/2885	*Canis lupus familiaris*	2022	Italy (Sicily, Palermo)	26	METK	-	*aqp1*	-	*b-cat, d-ldh*
MCAN/IT/2020/4299	*Canis lupus familiaris*	2020	Italy (Sicily, Palermo)	10	METK	-	-	-	-
MCAN/IT/2021/18017	*Canis lupus familiaris*	2021	Italy (Sicily, Palermo)	8	METK	-	*abcc2*	-	*b-cat*
MCAN/IT/2019/3299	*Canis lupus familiaris*	2019	Italy (Sicily, Palermo)	19	-	-	-	*LdMT*	-
MCAN/IT/2019/4549	*Canis lupus familiaris*	2019	Italy (Sicily, Palermo)	9	METK	-	*abcc1, abcc2, mrpa, ptr1*	-	-
MHOM/ES/2022/LLM-2505	*Homo sapiens sapiens*	2022	Spain (Toledo)	-	-	-	*aqp1*	-	-
MHOM/IT/90/ISS509	*Homo sapiens sapiens*	1990	Italy (Sicily, Catania)	-	-	-	-	-	*b-cat, d-ldh*
MHOM/IT/91/ISS666	*Homo sapiens sapiens*	1991	Italy (Sicily, Catania)	-	-	-	-	-	-
MHOM/IT/93/ISS828	*Homo sapiens sapiens*	1993	Italy (Sicily, Catania)	-	-	-	-	*LdRos3*	*b-cat*
MHOM/ES/2021/LLL-2500	*Homo sapiens sapiens*	2021	Spain (Madrid)	-	-	-	*abcc1, aqp1*	-	-
MCAN/IL/2011/NT6	*Canis lupus familiaris*	2011	Israel	-	METK	*smt2*	*abcc1, ptr1*	-	-

Drug-resistance biomarkers to antimonials: extended H-locus and the *aqp1* gene

Drug resistance biomarkers to antimonials were detected in 19 of the 35 *L. infantum* isolates through CNV detected in the *aqp1* gene and the extended H-locus. The biomarker for the *aqp1* gene was deemed resistant at <3.5 copies and was detected in 13 of the isolates. The biomarker for the extended H-locus was determined as resistant when >3.5 copies and was detected in 9 of the isolates. To calculate the extended H-locus copy number, copy number of the four genes within this region (*abbc1, abbc2, mrpa* and *ptr1*) was averaged. Individually, the biomarkers were detected as >3.5 copies in nine isolates for *abcc1,* eight for *abcc2*, nine for *mrpa*, and six for *ptr1*. Notably, the nine isolates with the presence of *mrpa* biomarker, also have the extended H-locus biomarker (>3.5 copies) and >3 copies of chromosome 23, highlighting the role of *mrpa* as an indicator of the entire locus in cultures grown without drug stressors.

For the 19 genetically resistant isolates, the following biomarkers profiles were detected: both extended H-locus and *aqp1* (4), extended H-locus only (5), *aqp1* only (8), *abcc1, mrpa* and *aqp1* (1), *abcc2* (1) (Table 2).

Drug-resistance biomarkers to allopurinol: METK locus

The drug-resistant biomarker for allopurinol was determined as <1.5 copies of *metk1,* <1.5 copies of *metk2*, or <3 copies for both *metk1* and *metk2*. The analysis showed that 13 out of 35 samples (37%) had at least one resistance biomarker. Considering the METK locus structure and the natural heterogeneity when losing one METK repeat unit, the METK locus (*metk1* plus *metk2*) was considered and analyzed as a single genomic resistance biomarker. The same 13 samples had a detectable METK locus resistance biomarker (<3 copies) when considered together. The results suggest that developing a technique enabling the quantification of both *metk* genes would be suitable for predicting allopurinol genomic resistance predisposition.

When considering both the canine and human isolates (n=35), 9 out of the 13 (69%) isolates with drug-resistance biomarkers to allopurinol also had resistance biomarkers to antimonials, whereas no isolate had genomic resistance predisposition to the combined therapy of allopurinol and miltefosine (Table 3). Similar results were obtained when focusing only on the canine host-derived isolates (n=22): seven out of the 11 (63%) positive samples for allopurinol were also resistant to antimonials. Conversely, as mentioned in the analysis of the general cohort, no culture had genomic resistance predisposition to the combined therapy of allopurinol and miltefosine.

Table 3. Genomic resistance predisposition to the combined allopurinol-antimonials treatment is the most frequent profile in allopurinol resistant *L. infantum* isolates. Summary of genomic resistance predisposition profiles to recommended combined therapies. Percentages are based on allopurinol-resistant isolates and remain consistent when analyzing the full cohort (n=35; allopurinol-resistant = 14) or only canine-derived cultures (n=22; allopurinol-resistant = 11).

Genomic resistance predisposition	Resistance biomarker	Samples (%)
Allopurinol + Antimonials	METK + extended H-locus/*aqp1*	63
Allopurinol + Miltefosine	METK locus + *LdMT*/*LdRos3*/MSL locus	0
Allopurinol only	METK locus	37

Drug-resistance biomarkers to amphotericin B: LdSMT locus

Genomic resistance biomarkers to amphotericin B are *smt1* and/or *smt2* genes when <1.5 copies, respectively, and were detected by LeishGenApp in four (13.8%) of the isolates (2 human and 2

canine derived isolates). Notably, one human isolated sample had both resistance biomarkers, while three samples exhibited a CNV exclusively to the *smt2* gene. Regarding canine isolates, both were at the threshold for biomarkers detection with a chromosome coverage close to quality inclusion criteria for LeishGenApp (MCAN/IL/2011/NT2: 1.33 copies, chr. cov =16; MCAN/IL/2011/NT6: 1.48 copies, chr. cov = 14).

The blastn alignment of the SMT region of the reference genome JPCM5 reported i) two identical copies of the *smt* gene: *smt1* and *smt2* in tandem (100% query coverage; 100% identity), ii) one repeated sequence of 1,268 bp with a direct orientation that includes one *smt* gene, along with 26 nucleotides at the 5' and 181 nucleotides at the 3' end of the gene. Sample reads were mapped to the reference genome JPCM5 identifying a 3,789 bp deletion (3,786-3,792 bp), corresponding to the combined length of the 1,268 bp repeated sequence and the unique intergenic sequence between the repeats (2,521 bp). This deletion is consistently observed in both samples with (<1.5 copies) and without (>1.5 copies) the genomic resistance biomarker, albeit in the ones without it, it occurs with minimal read frequency (≤ 0.5%).

Drug-resistance biomarkers to miltefosine: *LdMT* and *LdRos3* genes

Drug resistance biomarkers to miltefosine were detected in 3 of the 35 *L. infantum* isolates through CNV detected in the *LdMT* (2) and the *LdRos3* (1) genes. The biomarker for *LdMT* and *LdRos3* is determined as resistant when <1.5 copies (Table 2). *LdMT* encodes for a P-type ATPase phospholipid translocase, while *LdRos3* encodes for the β subunit of LdMT. Both proteins are part of the same flippase machinery, essential, and not individually sufficient for the translocation of miltefosine (Pérez-Victoria et al., 2006). Therefore, these three samples would be similarly resistant to miltefosine due to a decrease in drug accumulation, as they are defective in the uptake of miltefosine by being deficient in one of the required *LdMT* and *LdRos3* (Pérez-Victoria et al., 2006).

Drug-resistance biomarkers to paromomycin: *b-cat* and *d-ldh* genes

The drug resistance biomarker for paromomycin was determined as <1.5 copies of *b-cat,* and <1.5 copies of *d-ldh* (Table 2). Out of 35 samples, eight of them had at least one resistance biomarker, and, of these, four had both biomarkers. The prevalence of resistance biomarkers to paromomycin ranks as the third highest in the cohort (22.8%). While the prevalence in canine-derived isolates is low (13%), the prevalence in human isolates is notably higher (38%).

Structural analysis of the 15 genomic regions involved in drug resistance

In this study it was investigated whether exclusively local genomic rearrangements drifted the CNVs in key drug target and transporter genes or if the underlying aneuploidy might influence them. The analysis revealed three distinct correlation patterns when comparing the gene copy numbers of the eight loci of interest with the somy of their corresponding chromosomes.

The copy numbers of the extended H-locus and *LdMT* showed a strong correlation (0.88, p-val < 0.001 for H-locus; 0.76, p-val < 0.001 for *LdMT*) with their respective chromosome somy (23 and 13, respectively), suggesting that aneuploidy events drive these CNVs. Contrarily, *LdRos3,* LdSMT, METK, and PMM locus copy numbers were independent of their respective chromosome somy status, with either a null or low correlation (Supplementary Figure 1). This opposite behavior suggests that the change in these loci copies results from local genomic rearrangements. Lastly, moderate correlations were observed for *AQP1* and MSL loci on

tetrasomic chromosome 31, suggesting a more complex interaction, possibly a mix, between chromosomal and sub-chromosomal events contributing to CNVs.

Genomic structure of the extended H-locus and the METK locus were thoroughly analyzed. These biomarkers were specifically selected because i) they have opposite correlation patterns and ii) they are the most prevalent resistance biomarkers in our cohort.

Genomic organization of the extended H-locus region

As defined above, we considered an extended H-locus comprising the *abcc1*, *abcc2*, *mrpa,* and *ptr1* genes. The genome sequence between coordinates 59,772 to 106,565 of chromosome 23 of *L. infantum* JPCM5 reference was aligned to itself using both Nucmer and blastn. Repeated sequences were filtered for lengths above 0.5kb, identifying three distinct repeated sequences. While analyzing the 35 *de novo* assembled genomes, we successfully identified these three sequences that uphold the locus's conserved structure across various *Leishmania* isolates, that can be the base for recombination events and thus support extrachromosomal elements formation. Firstly, a 1.44 kb intergenic sequence repeated three times (Rep01.01, Rep05.01 and Rep06.01) with >99.5% nucleotide identity: Rep01.01 with inverted orientation, inverted repeat (IRs), whereas Rep05.01 and Rep06.01 are in direct orientation, direct repeats (DRs) (Figure 3). Similar repeats have been previously described (Leprohon et al., 2009 and Ubeda et al., 2014). The DR Rep05.01-Rep06.01 could generate a 15.2kb circular episome produced by Homologous Recombination (HR) containing *LinJ.23.0270*, *yip1*, *mrpa*, and *LinJ.23.0300* genes, or the annealing of the IRs Rep01.01-Rep05.01 and Rep01.01- Rep06.01 could be the base of a 172kb or 186kb linear mini-chromosomes formation, respectively.

Secondly, a 0.5kb sequence with 85% identity corresponding to *ATP binding cassette* domain, and thirdly, a 0.9kb sequence with 90% identity corresponding to an *ABC transporter integral membrane type-1 fused domain* and *ATP binding cassette* conserved domains, both shared by *abcc1*, *abcc2*, and *mrpa* genes.

We did not detect any of these extrachromosomal elements in the *de novo* assembled genomes of our cohort, the gene copy gains or loss of the extended H-locus is correlated with the chromosome 23 somy.

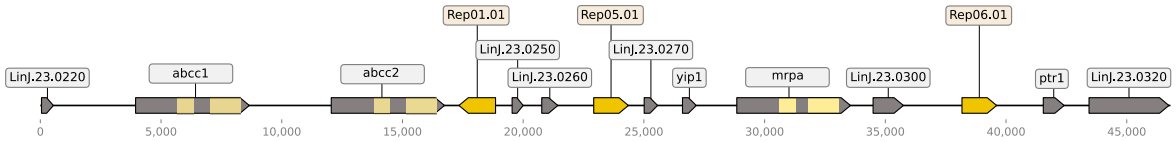


Figure 3. Description of extended H-locus structure in *L. infantum* and within the cohort. a) Genomic organization of extended H-locus in *L. infantum*. Genes are indicated in grey and intergenic repeated sequences in orange (DR, Rep05.01 and Rep06.01; IR, Rep01.01). Homologous regions between genes are shown in light orange.

Copy number variation in the METK locus

CNV within the METK locus (chr. 30) was described in 14 out of the 35 (40%) isolates. This CNV, led by a deletion, spans 5.1kb (range from 5,106 to 5,136 bp). In the reference strain JPCM5 (RefSeq: GCF_000002875.2 ASM287v2), the METK locus comprises two identical copies of

5,120 bp (Rep.01.01, Rep01.02). Each repeat unit contains a Lorien protein coding gene *(LinJ.30.3550/LinJ.30.3570*) and a *metk* gene (*LinJ.30.3560/LinJ.30.3580*) encoding for an S-adenosylmethionine synthetase. Both Lorien proteins and the *metk* genes showed 100% query coverage with 100% identity. Additionally, as shown in Figure 4, the flanking regions of the four genes share 218 identical bp, further confirming the duplication's structural integrity (Rep02.01 Rep02.02, and Rep02.03).

When mapping sequencing reads from the 15 samples to the JPCM5 reference genome, no precise breakpoint for the 5.1kb deletion was detected but the loss of one of the two constructs was consistently identified. The CNV always occurred on a disomic background, suggesting the samples are hemizygous for the construct. However, due to the sequence identity of flanking regions, it was not possible to determine whether the deletion occurred in the first or the second repeat unit on each chromosome. When sequencing reads were mapped from the 14 samples to their corresponding *de novo* assembled genome, conversely, no evidence of deletions was observed as the reads and coverage were continuous along the whole region. Reads had a single copy of the 5,120bp repeat unit, which encodes for a single Lorien protein coding gene and a *metk* gene, as corroborated by the annotation of the assembled genomes.

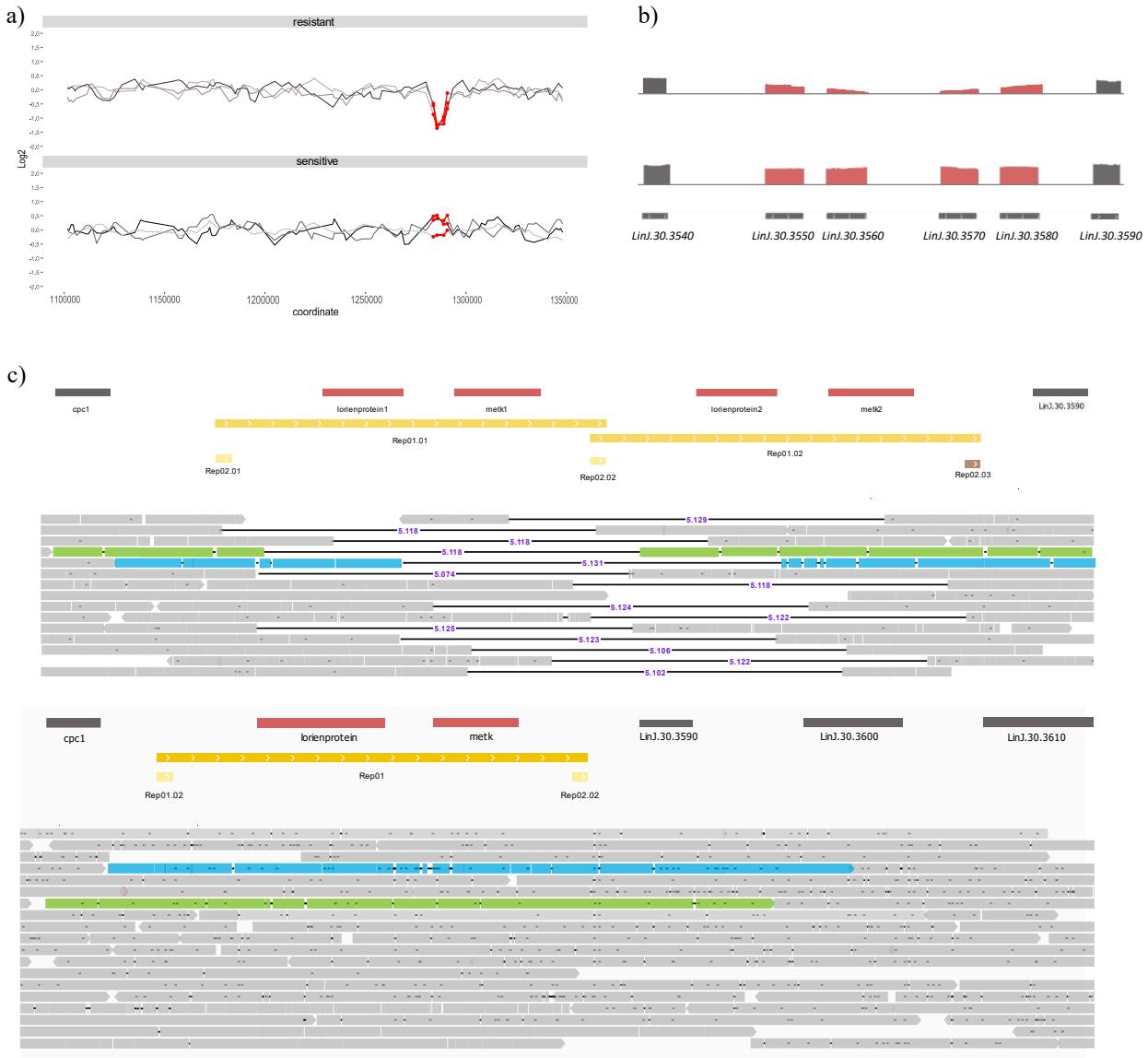


Figure 4. Characterization of a 5.1kb deletion in the METK locus region of *L. infantum* a) Log_2 transformed normalized reads for non-overlapping 1kb genomic window on chromosome 30 (1100000 ...1350000) for deletion and non-deletion cultures. Red, METK locus (LinJ.30.3550, LinJ.30.3560, LinJ.30.3570, and LinJ.30.3580). b) The coverage in the METK locus area is decreased due to gene loss, unlike the even coverage seen in samples with both constructs. c) On top, the METK locus region and the DR repeats (dark yellow). DR are 01.01-01.02 and 02.01-02.02-02.03. A subsampling of reads from a genomic allopurinol-resistant sample mapped against the reference genome does not reveal a clear breakpoint for the 5.1kb deletion. Underneath, mapping of the same subsampled reads to the isolate's *de novo* assembly. Reads were continuous as only one 5,120bp construct was present. The different behavior of the same reads (green and blue) when mapped against the reference genome (top) or its assembly (bottom) evidenced the presence of a CNV.

Discussion

Detection of aneuploidies and genome plasticity in *L. infantum* genome by nanopore-only whole-genome sequencing

The genome of *L. infantum* exhibits high plasticity, as evidenced by aneuploidy, CNVs, and Single Nucleotide Polymorphism (SNP) (Leprohon et al., 2015). Previous studies assessing read depth coverage from next-generation sequencing data have shown the widespread occurrence of supernumerary chromosomes (Franssen et al., 2020; Negreira et al., 2022), confirming that aneuploidy is more prevalent in *Leishmania* than initially expected, being common even in cells not subjected to any stress besides culturing (Rogers et al., 2011).

Our results demonstrate the feasibility of uncorrected nanopore long reads to detect aneuploidy, including intrastrain mosaicism and interstrain diversity, revealing extensive variation in chromosome copy number among the 35 whole-genome sequenced isolates of the cohort. Some chromosomes have a higher propensity for aneuploidy turnover than others, and despite some minor discrepancies, these findings are broadly consistent with those reported in a study with 161 cultured samples (Franssen et al., 2020). Results also agree that five chromosomes (19, 27, 28, 34, and 36) showed almost no variation, and two have high copy number variation (8 and 23) (Franssen et al., 2020). Based on the median chromosome copy number, trisomy was identified in chromosomes 8, 20, and 23, consistent with previous reports (Franssen et al., 2020), which described chromosomes 8 and 23, besides 9, as trisomic. Contrary, monosomy was scarce, a finding also corroborated by the same authors (Franssen et al., 2020).

The present data cannot demonstrate a significant effect of mid-term culture stress on the emergence of aneuploidies for isolates of up to 33 passages, suggesting that the primary factor contributing to aneuploidy is the inherent genomic instability of the *Leishmania* strain rather than the culturing pressure itself. That is advantageous for clinicians, as it demonstrates that the *in vitro* isolate is similar to the *Leishmania* population in the infected human or dog. However, the data suggests a possible effect of the extreme *in vitro* number of passages, as the long-established reference line MHOM/TN/80/IPT-1 had 10 supernumerary chromosomes. Further investigation, comparing the clinical sample with its derived serial passages, into the role of culture-induced stress in accelerating aneuploidy may be of interest to better understand its impact on genomic stability and help establish a clear threshold for the passage effect. Furthermore, a study of the potential effects of different culture media on aneuploidy induction may be complementary.

Leishmania is emerging as a unique model for aneuploidy and karyotype heterogenicity in mosaic aneuploidy, where individual cells in a population may display distinct somies (Negreira et al., 2022). The results suggest that every chromosome analyzed was shown to be present in at least two ploidy states in the population and emphasize the importance of single-cell studies to uncover the individual karyotype (Sterkers et al., 2011;Imamura et al., 2020; Negreira et al., 2022), as

mosaic populations in untreated isolates harboring stochastic variations may gain a selective advantage when exposed to drugs, driving resistance development.

In the context of drug resistance, aneuploidy was first observed in chromosomes 1, 11, and 25 in antimonial-resistant mutants (Leprohon et al., 2009). The authors described an increase of entire chromosomes DNA content, correlating well with mRNA profile and gene overexpression (Leprohon et al., 2009). Cultures MHOM/MA/67/ITMAP-264 and MHOM/MA/67/ITMAP-265 of the present cohort were polyploid for chromosomes 1 and 11. Interestingly, both cultures were derived from the same initial population, but the latter underwent 10 additional passages. According to the genomic resistance profile predicted at the chromosomal level, gene CNVs were detected as antimony resistance biomarkers in MHOM/MA/67/ITMAP-264 *(abcc1*, a*bcc2*, and *mrpa*) and in MHOM/MA/67/ITMAP-265 (*abcc1*, *abcc2*, *mrpa*, and *aqp1*). However, no aneuploidy for chromosomes 1, 11, or 25 was found in any other cultures from our cohort where CNVs described as antimonial resistance biomarkers were identified Leprohon and collaborators (2009) observed that the withdrawal of drug pressure led to the reversion of supernumerary chromosomes 1, 11, and 25 to their original somy levels. Their findings suggest none of these chromosomal adaptations were actively developed in our isolates, as none of our cultures were exposed to drug pressure during culturing.

CNV for drug resistance biomarkers in *L. infantum*

The present study also investigated whether exclusively local genomic rearrangements drift the CNVs in key drug target and transporter genes or if they are influenced by the already discussed aneuploidy, detecting three different correlation patterns.

Of interest, the chromosome 23 somy showed a strong positive correlation with the copy number of the extended H-locus genes, suggesting that aneuploidy events are drivers of these genes' CNVs, with nine isolates having >3 copies of both chromosome 23 and extended H-locus. Although described as a highly unstable chromosome, *L. infantum* isolates with up to 3 copies of chromosome 23 are known to be sensitive to antimonials without extrachromosomal amplifications (Dumetz et al., 2018). Based on the evidence from the literature, the genomic resistance biomarkers threshold was set as >3.5 copies for the four extended H-locus genes in chromosome 23, contrary to other biomarkers encoded in disomic chromosomes (see Table 1). Chromosome 23 consistently displayed an increase in copy number in *in vitro* populations selected for antimonial resistance in which *mrpa* was artificially deleted (Negreira et al., 2023) suggesting an adaptive advantage as it is known as a rapid way to amplify a cassette of genes involved in the antimonials pathway. The results of this study pointed out that, beyond the intrinsic variability of the chromosome, the widespread increase in the dosage of chromosome 23 observed in the cohort may represent a form of pre-adaptation to trivalent antimonials, likely driven by the sustained antimonial pressure exerted overuse decades in the Mediterranean basin (Haldar et al., 2011).

Gene CNVs play a crucial role in the parasite's adaptability to drug stress through gene amplification or expansion and gene deletion, an event driven by widely distributed repeated sequences, typically found in non-coding regions, leading to stochastically genome rearrangements (Ubeda et al., 2014; Prieto Barja et al., 2017). Gene amplifications are generally extrachromosomal and generated by HR between DR or hairpin formation between IR (Leprohon et al., 2009; Ubeda et al., 2014). Similar repeated sequences flanked the two in-depth studied loci (extended H-locus and METK locus), providing structural flexibility for potential rearrangements.

The three repeated sequences described in the extended H-locus suggest the potential formation of 4 circular episomes and two linear minichromosomes, depending on the stochastic rearrangement. However, no episomal amplification was detected in our cohort. The promastigote cultures analyzed were grown under natural conditions. In contrast, experimental episomal amplification has mainly been detected in cultures grown under drug pressure and demonstrated that it generally disappears rapidly when the drug pressure is removed (Beverley et al., 1984) (Ubeda et al., 2008) (Leprohon et al., 2009) (Ubeda et al., 2014). On the other hand, we cannot discard the effect of the DNA extraction procedure used as a drawback to episomal recovery.

Concerning allopurinol resistance, a 5.12 kb repeated sequence flanked by 218 bp repeats was detected on chromosome 30 in the METK locus. This 218 bp intergenic repeated sequence may have been a determinant in *L. infantum* METK locus structure rearrangement, as seen on the reference genome and 21 samples (containing two identical 5.12 kb copies in tandem). That 218 bp repeat might be sufficient in length for the loss of a 5.12kb METK region by HR, similar to the 220bp direct repeat at the start codon of the tartrate-sensitive acid phosphatase (*LdBPK_366740*) and histidine secretory acid phosphatase (*LdBPK_366770*) that enables the MAPK locus gene amplification in antimonial resistant lines, maintained as a 1,700bp episomal DNA (Downing et al., 2011). Moreover, a minimum identity of 85% is required for recombination in *Leishmania* (Coelho et al., 2012) and we described a 97-100% identity. Therefore, the METK and the SMT locus studied are examples of gene amplification strategies, as two identical copies are in tandem.

Prevalence of genomic resistance to the five first-line antileishmanial drugs

From a Public Health perspective, LeishGenApp is a powerful tool for monitoring drug-resistant *Leishmania infantum* strains circulating in both human and canine populations. Particularly in dogs, highlighting their role as primary reservoirs, lessening clinical relapses due to resistance, and their implications in sand fly transmission. Differences in genomic resistance biomarkers prevalence in our cohort underscore the importance of targeted drug policies to mitigate resistance dissemination. Miltefosine resistance was notably low (9%), likely due to two factors: i) miltefosine-resistant parasites exhibit reduced fitness in mammalian hosts limiting their spread, despite maintaining their phenotype through the vector (Van Bockstal et al., 2020), and ii) miltefosine is not used in HumL treatment, reducing selective pressure (Gradoni et al., 2017). These findings support WHO recommendations to restrict miltefosine use to CanL, preventing resistance emergence in humans while preserving its efficacy in dogs. Conversely, antimonial resistance showed the highest prevalence (canine: 50%; human: 61%). Resistant parasites retain their phenotype through sand flies and demonstrate enhanced fitness compared to sensitive strains (Ouakad et al., 2011), being a substantial thread in areas where antimony drug pressure is sought to be low. This underscores the need for careful regulation of antimonial use to control resistance spread. For paromomycin, the observed resistance prevalence was higher in humans (38%) than in dogs (14%). Resistant parasites maintain their phenotype through sand flies (Hendrickx et al., 2020), but reduced fitness in the absence of drug pressure may explain its limited dissemination in CanL.

Concerning CanL, allopurinol is the primary drug for the long-term management of cases (LeishVet, 2018). Resistance biomarkers to this drug were detected in 50% of the canine isolates. This study aimed to investigate a potential link between the recommended combined therapy for the management of CanL, which includes a leishmaniostatic drug (allopurinol) and a leishmanicidal drug (meglumine antimoniate/miltefosine), and the natural emergence of genomic resistance in *L. infantum* field isolates. Interestingly, the present data showed that among the isolates having allopurinol resistance biomarkers, the highest proportion have genomic resistance biomarkers to the combined therapy of antimonial and allopurinol, aligning well with the earliest

use of these drugs (Baneth & Shaw, 2002). On the other hand, our results showed no isolates showing genomic resistance biomarkers to the combined therapy of allopurinol and miltefosine. We detected only one *in vitro* clinical isolate with biomarkers of miltefosine resistance (MCAN/IT/2019/3299). Sample 3299 was isolated in 2019 after the acceptance of miltefosine for CanL treatment. Knowing whether this canine subject had been treated with miltefosine before sample collection would be valuable as miltefosine has a long half-life in the treated host, possibly leading to the retention of sub-therapeutic levels in the body (Pérez-Victoria et al., 2006), which may trigger the development of resistance (Gonçalves et al., 2021). The low miltefosine resistance biomarkers prevalence aligned with the later approval for miltefosine to CanL treatment in Europe (2007) and the few existing clinically resistant cases in the literature, the first reported in 2020 (Gonçalves et al., 2021).

Treatment guidelines for CanL recommend combined therapy with at least two drugs with different mechanisms of action to delay drug resistance (Bryceson, 2001; van Griensven et al., 2024). Our findings support the effectiveness of this strategy, as 50% of canine isolates showed resistance biomarkers to only one drug. However, resistance to combination therapy is still possible, with 31% of canine isolates showing genomic resistance to two or three drugs, consistent with previous reports (García-Hernández et al., 2012). In contrast, for HumL, monotherapy is the recommended treatment approach (Gradoni et al., 2017). In human isolates, resistance to two drugs is the most common profile (38%), suggesting a potential impact of therapeutic strategies on resistance emergence. Of concern, a possible preadaptation to antimonials due to drug pressure in old-world *Leishmania* circulating in humans may act as a confounding factor, as 80% of isolates resistant to two drugs carried biomarkers linked to antimonial resistance (Haldar et al., 2011; Dumetz et al., 2018). Further clinical trials on combination therapy for HumL in the Mediterranean region are warranted to clarify the influence of therapeutic strategies on resistance profiles.

Concerning HumL, genomic resistance biomarkers to antimonials were most prevalent, followed by resistance to paramomycin. These findings are in line with the current treatment guidelines for both visceral leishmaniosis (pentavalent antimonials and liposomal amphotericin B as first-line treatments) and cutaneous leishmaniosis (albeit their late approval (2006) paromomycin ointment and intralesional antimonials as first topical treatments; and pentavalent antimonials as first systemic treatment) caused by *L. infantum* in the Mediterranean basin (Gradoni et al., 2017). Of particular interest, our research revealed an interestingly low prevalence of genomic resistance biomarkers to amphotericin B (15%). This underscores the importance of the WHO recommendations for the European veterinarians to avoid using this drug, particularly as efficacy is not completely clear in dogs (Hernández et al., 2015), and they may develop higher fitness-resistant parasites (Mano et al., 2023) that can be transmitted to humans. Also, the low prevalence of genomic resistance to allopurinol in HumL when compared with CanL isolates is noteworthy given that allopurinol is not used in HumL treatment (Gradoni et al., 2017).

Our findings on the prevalence of CNVs as genomic resistance biomarkers in *L. infantum* isolates support the clinical significance of LeishGenApp in improving patient management. This approach enables timely and precise therapy by accurately identifying resistant parasites, thereby reducing drug-related morbidity and curbing resistance spread. Resistant *L. infantum* strains in canine subjects, the primary host and main reservoir, are of concern as they may enhance uncontrolled transmission to other dogs and even humans, hindering this zoonotic infection progress. Expanding the spectrum of gene CNVs described as genomic resistance and pathogenicity biomarkers, combined with the detection of single point mutations associated with drug resistance, would enhance LeishGenApp accuracy, enabling better patient management

concerning combined therapy administration. In addition, conducting molecular *in vivo* analysis (i.e., inhibitory concentrations (IC50)) of the studied cultures may be of interest to establish a robust correlation between the genomic resistance biomarkers identified through WGS and the phenotypic traits.

LeishGenApp could help in the early characterization of stochastic aneuploidy variations, which can provide a selective advantage under drug pressure, and detect chromosomal and sub-chromosomal genomic resistance biomarkers. The implementation of this framework can guide therapeutic strategies, delay resistance development, and enhance genomic surveillance to mitigate the risks of relapses and drug-resistant subpopulations in both canine and human leishmaniosis. Therefore, integrated surveillance following the One Health approach must be strengthened to improve disease control. By enabling the selection of cases based on their genomic resistance profiles, LeishGenApp helps delay the emergence of resistance, prolonging the therapeutic lifespan of current drugs.

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