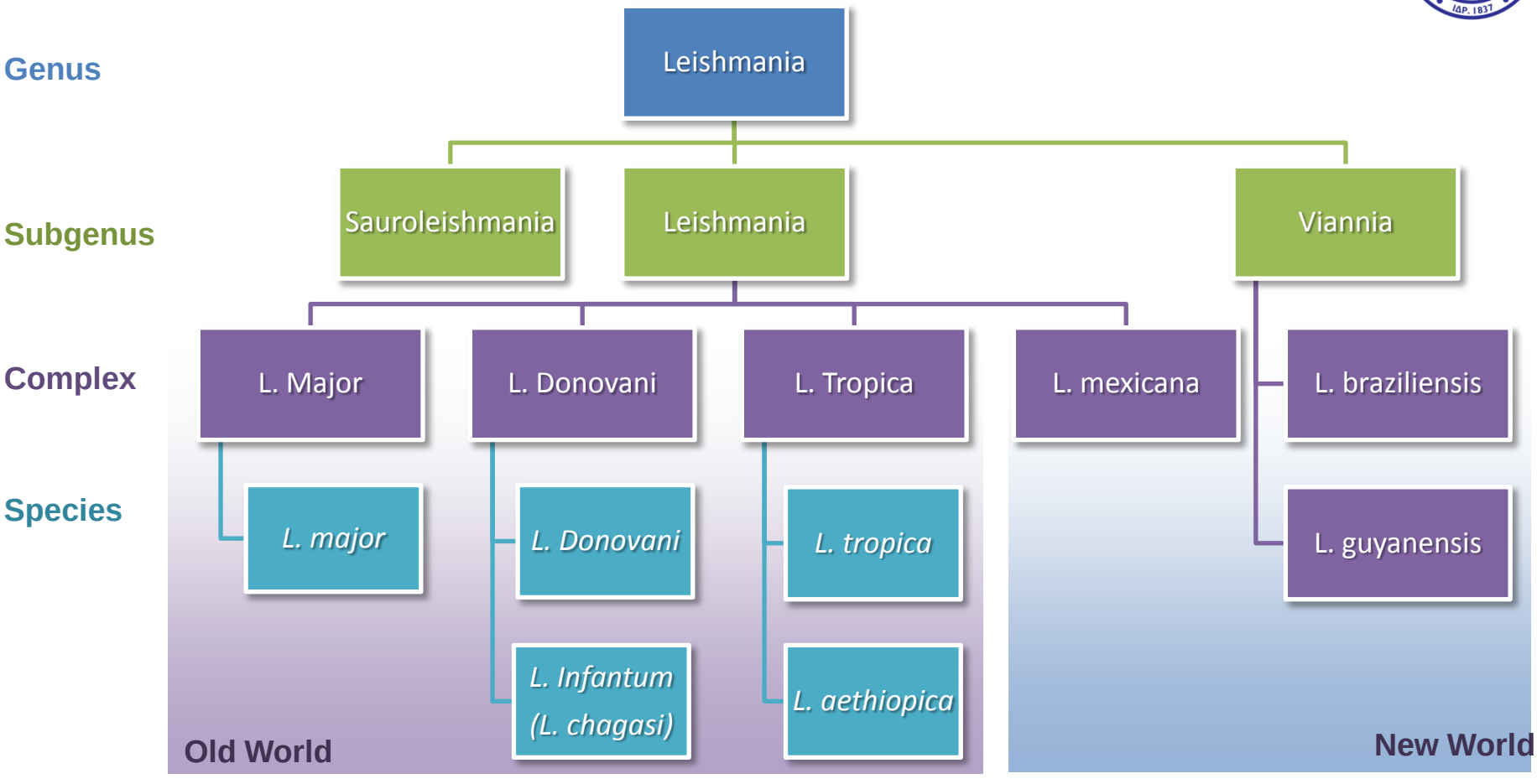
ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΙΑΤΡΙΚΗ ΣΧΟΛΗ
Α' ΠΑΘΟΛΟΓΙΚΗ ΚΛΙΝΙΚΗ

Διευθυντής: Καθηγήτρια Ε.Ι. Γκόγκα

Leismaniasis: approach to diagnosis and epidemiology with molecular methods

M. Samarkos
Associate Professor in Medicine – Infectious Diseases

Leishmania taxonomy

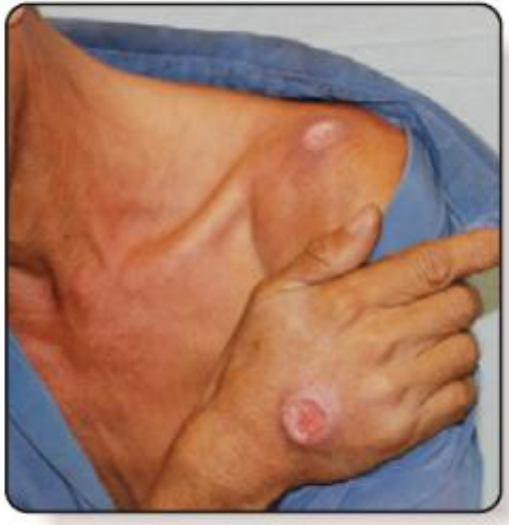


Leishmaniasis

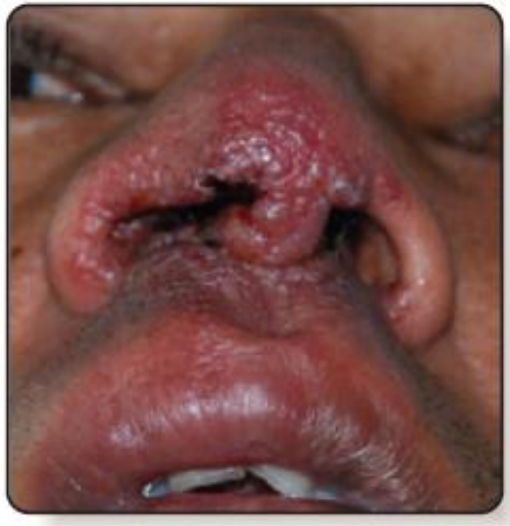
- Leishmaniases are vector-borne parasitic diseases caused by at least 20 species of the genus *Leishmania.*
- Transmission between mammalian hosts by female sandflies.
- Primarily zoonotic with the exception of *Leishmania donovani* and *Leishmania tropica*, although there is some evidence that animal reservoirs exist for both species across Africa and Asia
- Distinct species of *Leishmania* cause different clinical manifestations, ranging in severity from self-curing cutaneous lesions to life-threatening visceral disease.
- Neglected tropical disease according to WHO (2017)

Lancet 2018; 392: 951–70

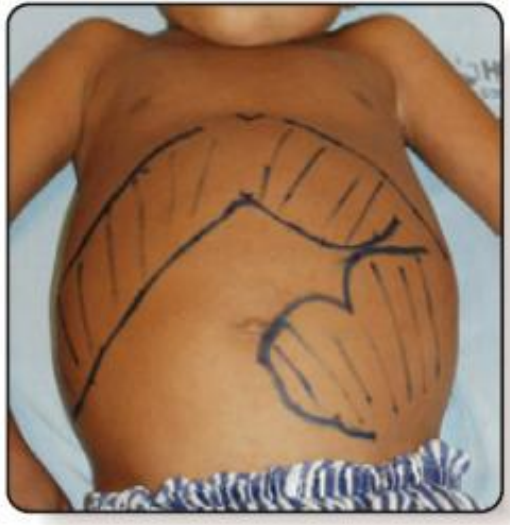
Leishmaniasis – Clinical forms



Cutaneous
Variable chronic ulcer with raised borders



Mucosal
Erosion of nose, lips, palate, cheeks, pharynx or larynx



Visceral
Hepatosplenomegaly, fever, malnutrition, neutropenia, hypergammaglobulinemia

Tropical infectious diseases: principles, pathogens and practice. Ch 100, 3rd ed (2011).

Pathogenic leishmanias

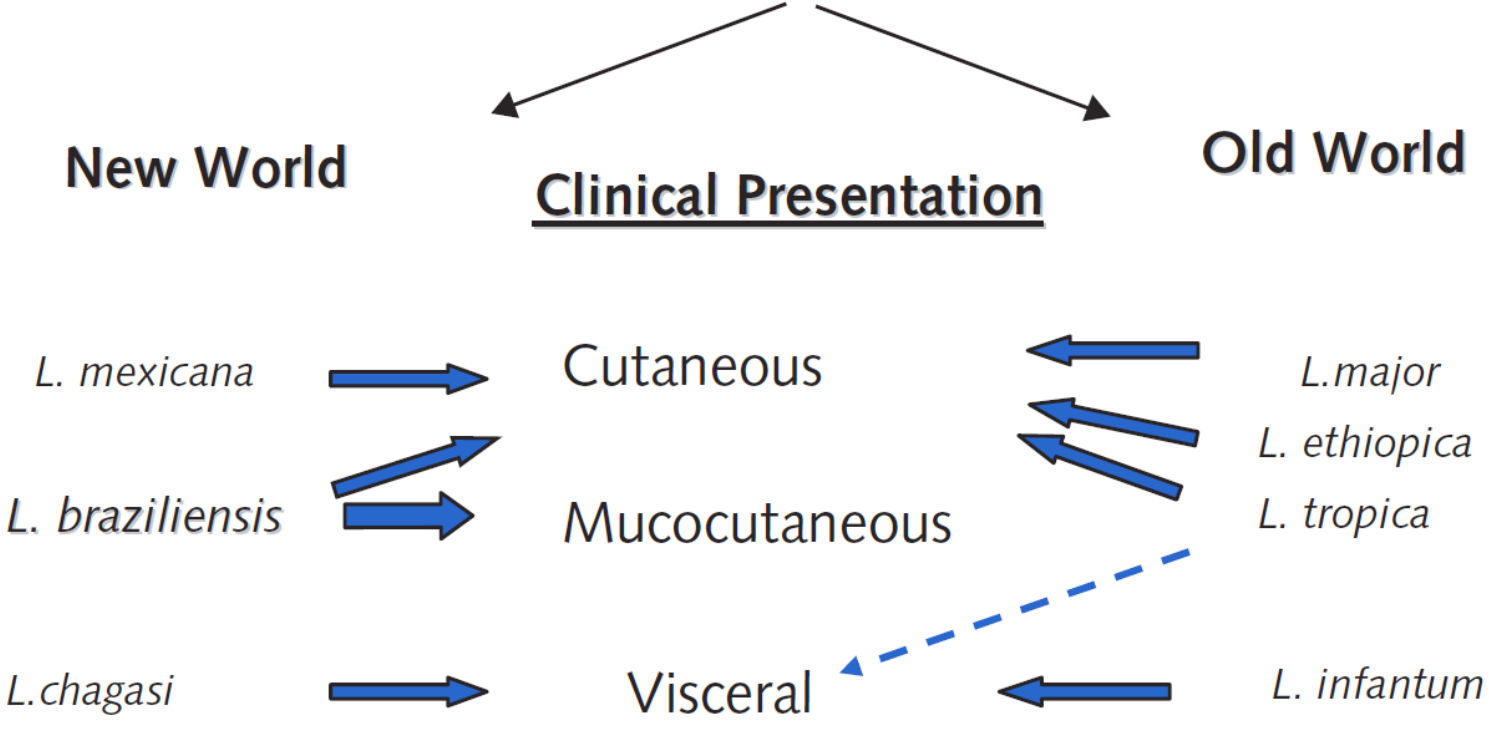
TABLE 47.1 ***Leishmania* Species Found in Humans**

Subgenus	*L.* (*Leishmania*)	*L.* (*Leishmania*)	*L.* (*Viannia*)	*L.* (*Viannia*)
Old World	L. *donovani* L. *infantum*	L. *major* L. *tropica* L. *killicki*[a] L. *aethiopica*		
New World	*L. infantum*	L. *infantum* L. *mexicana* L. *pifanoi*[a] L. *venezuelensis* L. *garnhami*[a] L. *amazonensis*	L. *braziliensis* L. *guyanensis* L. *panamensis* L. *shawi* L. *naiffi* L. *lainsoni* L. *lindenbergi* L. *peruviana* L. *colombiensis*[b]	L. *braziliensis* L. *panamensis*
Principal tropism	Viscerotropic	Dermotropic	Dermotropic	Mucotropic

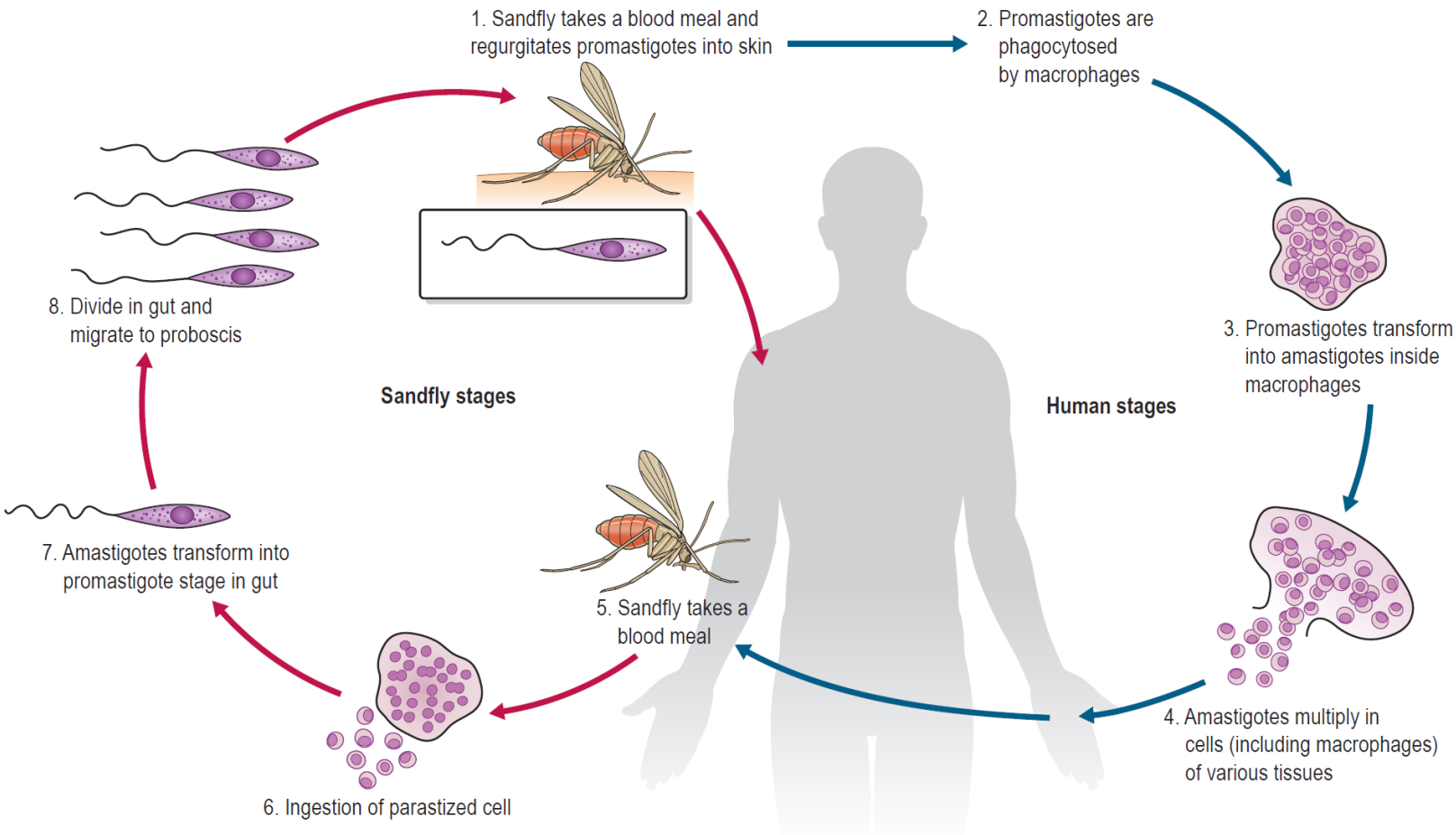
[a]Species status is under discussion.
[b]Taxonomic position is under discussion.
Source: World Health Organization. Control of the Leishmaniases. Geneva: WHO press. WHO Technical Report Series 2010;949:1–186. Reproduced with permission.

Manson's Tropical Diseases, 23rd Ed (2014)

Leishmaniasis – Clinical forms



Leishmania life cycle



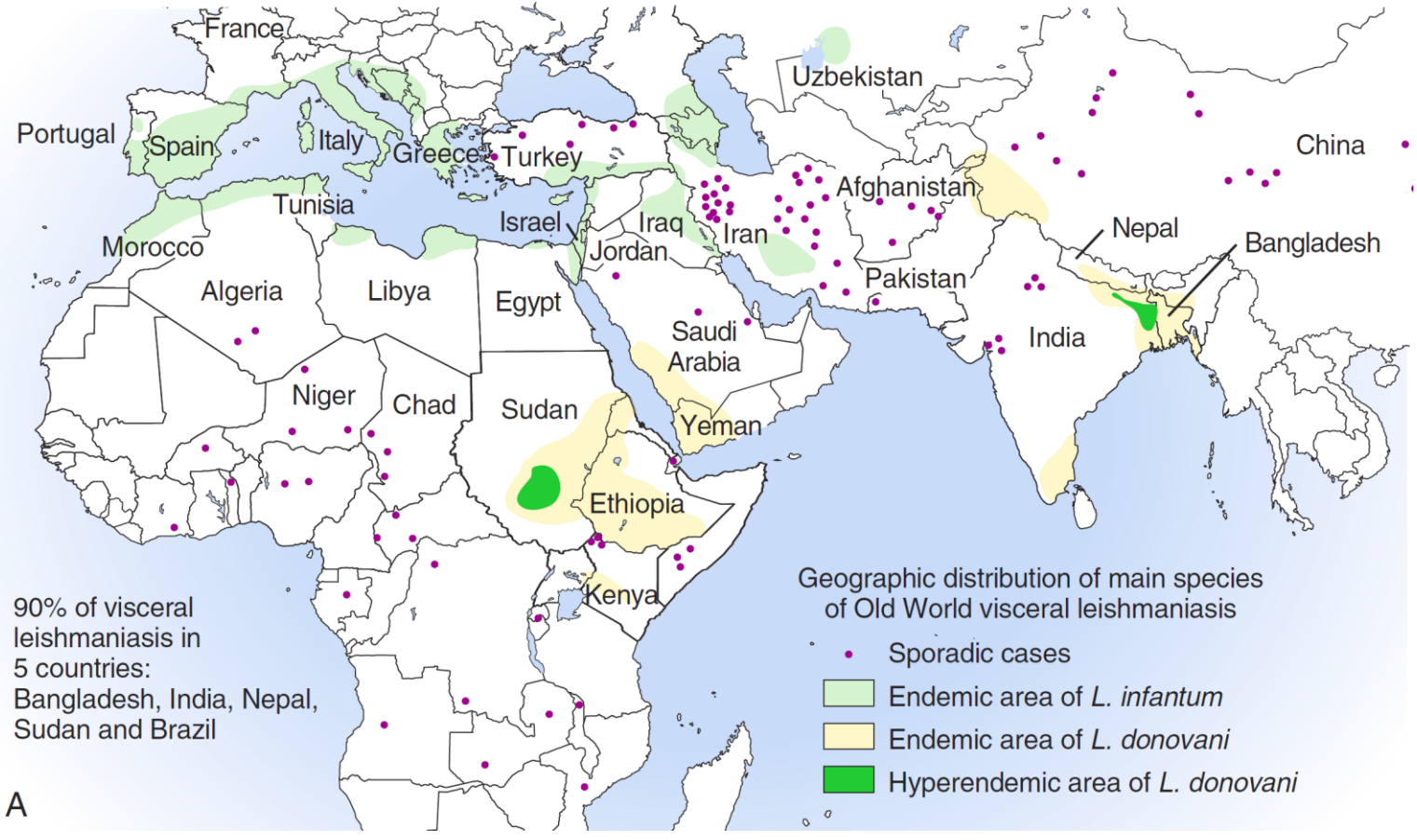
Lancet Infect Dis 2007;7:581–96.

Epidemiology of visceral leishmaniasis

Epidemiology of VL

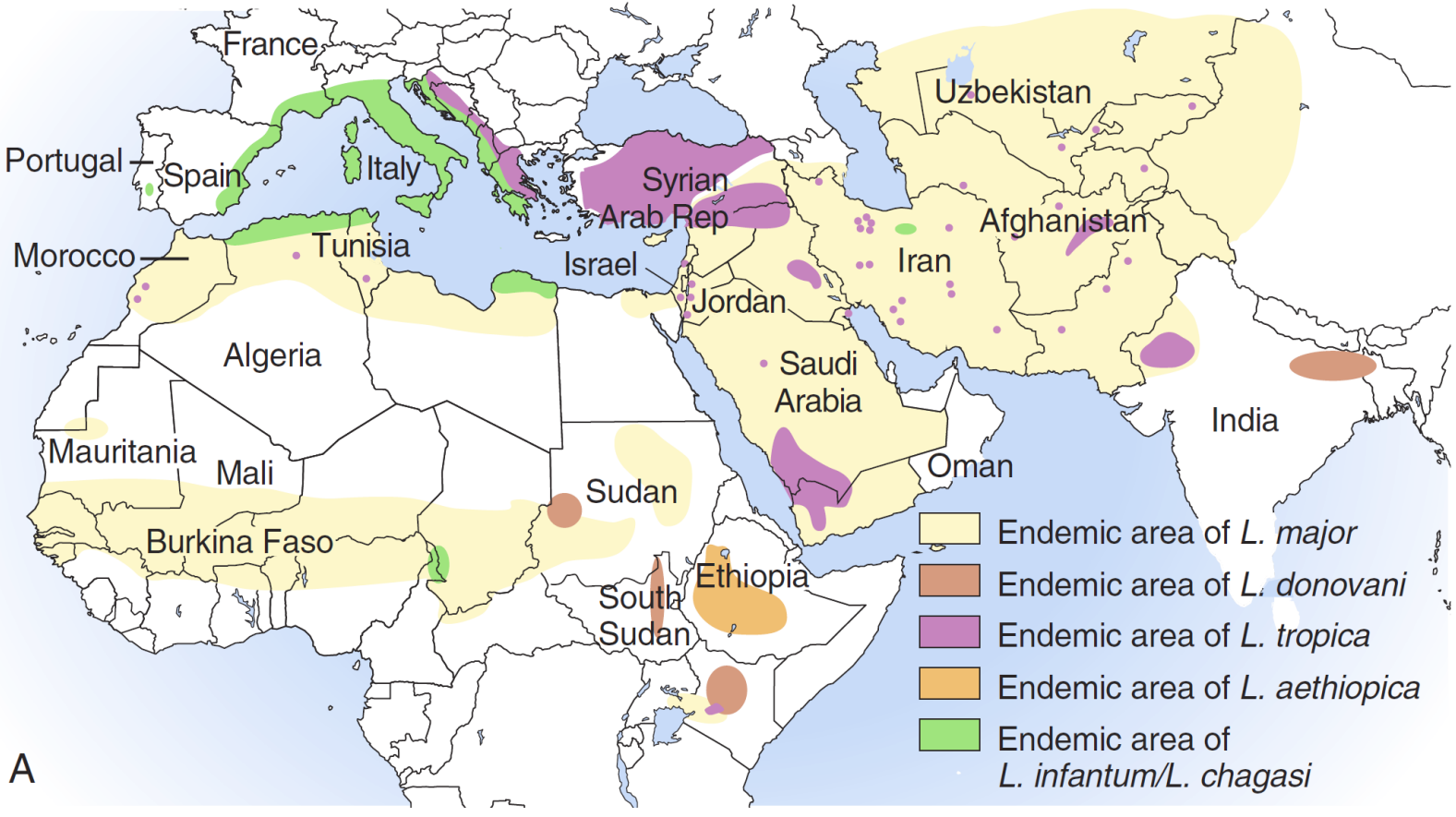
- The disease remains endemic in more than 60 countries.
- In 2015, seven countries (Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan) reported more than 90% of the worldwide cases of visceral leishmaniasis.
- The global incidence of visceral leishmaniasis decreased substantially in the past decade: from between 200 000 and 400 000 new cases in 2012, to between 50 000 and 90 000 in 2017.
- Asymptomatic infection is common in endemic areas.
  - Seroprevalence: 7% - 63% for L donovani in the Indian subcontinent, 29% -34% for L infantum in Brazilian children.
  - 17% PCR positivity in a cohort >4000 asymptomatic people in Ethiopia

Geographic distribution of main species of Old World visceral leishmaniasis.



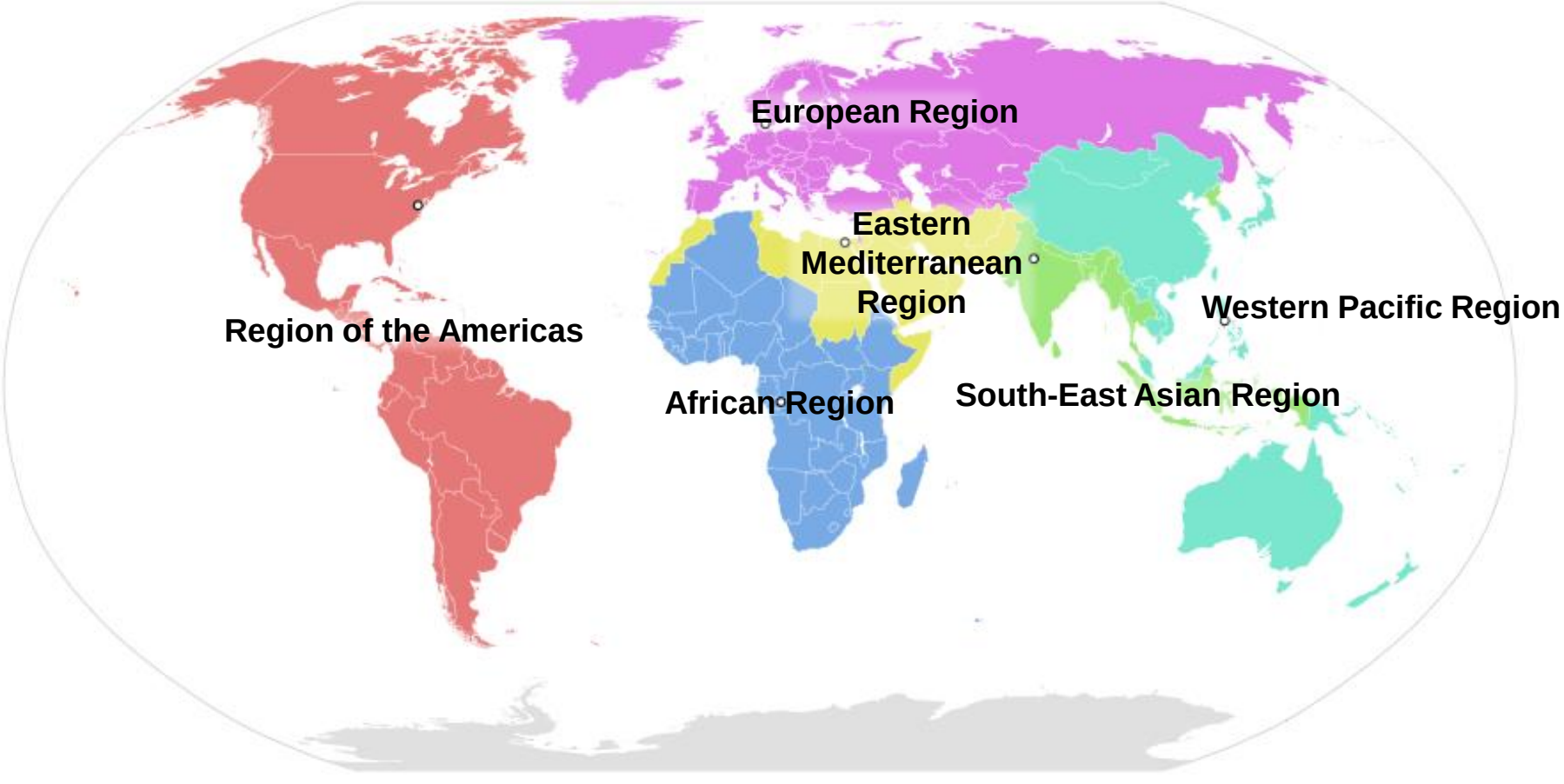
Principles & Practice of Infectious Diseases, Ch 277, 8th Ed (2015)

Geographic distribution of main species of Old World cutaneous leishmaniasis.

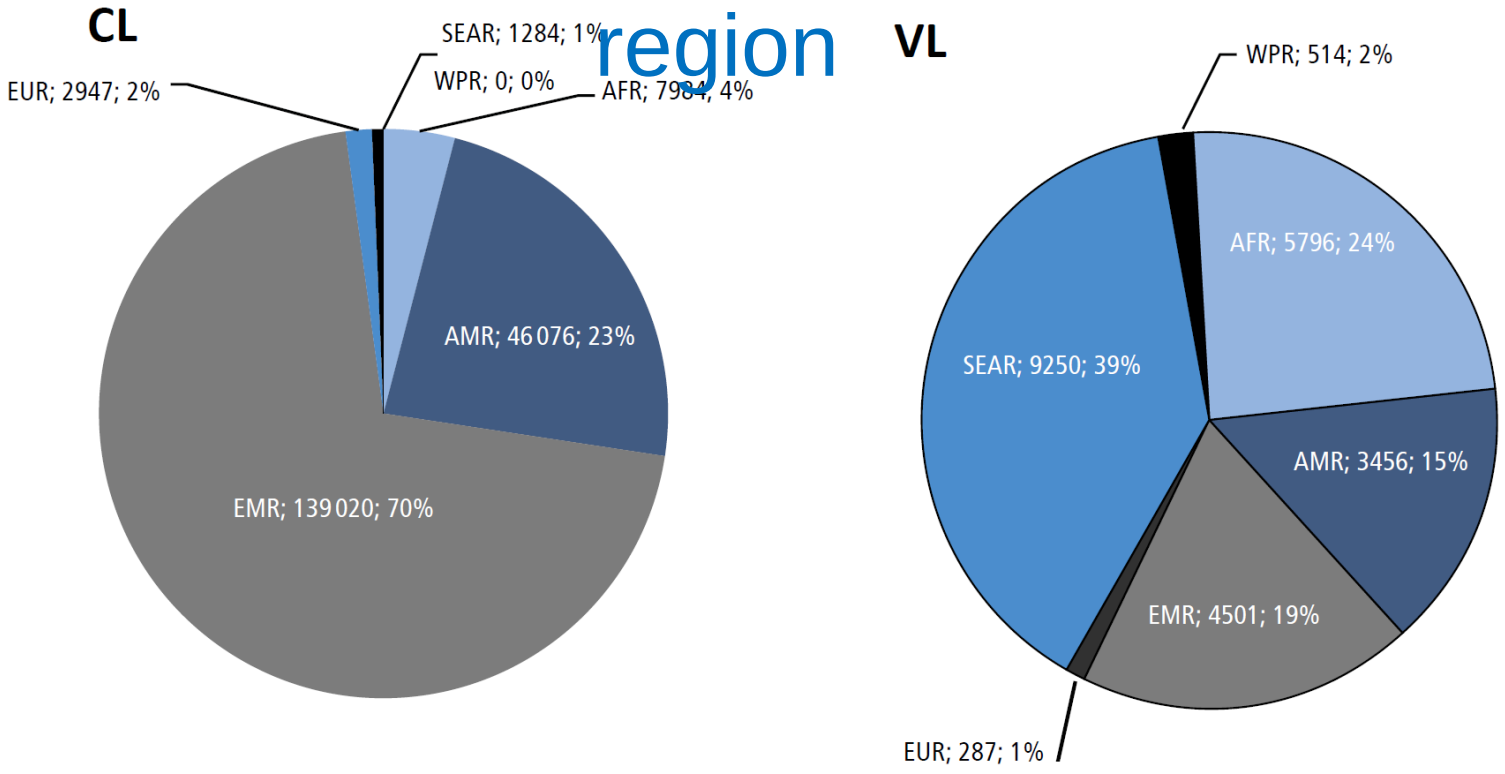


Principles & Practice of Infectious Diseases, Ch 277, 8th Ed (2015)

WHO Regions


European Region
Eastern Mediterranean Region
Western Pacific Region
Region of the Americas
African Region
South-East Asian Region

Number of new cases of CL and VL reported to WHO in 2015, by WHO region

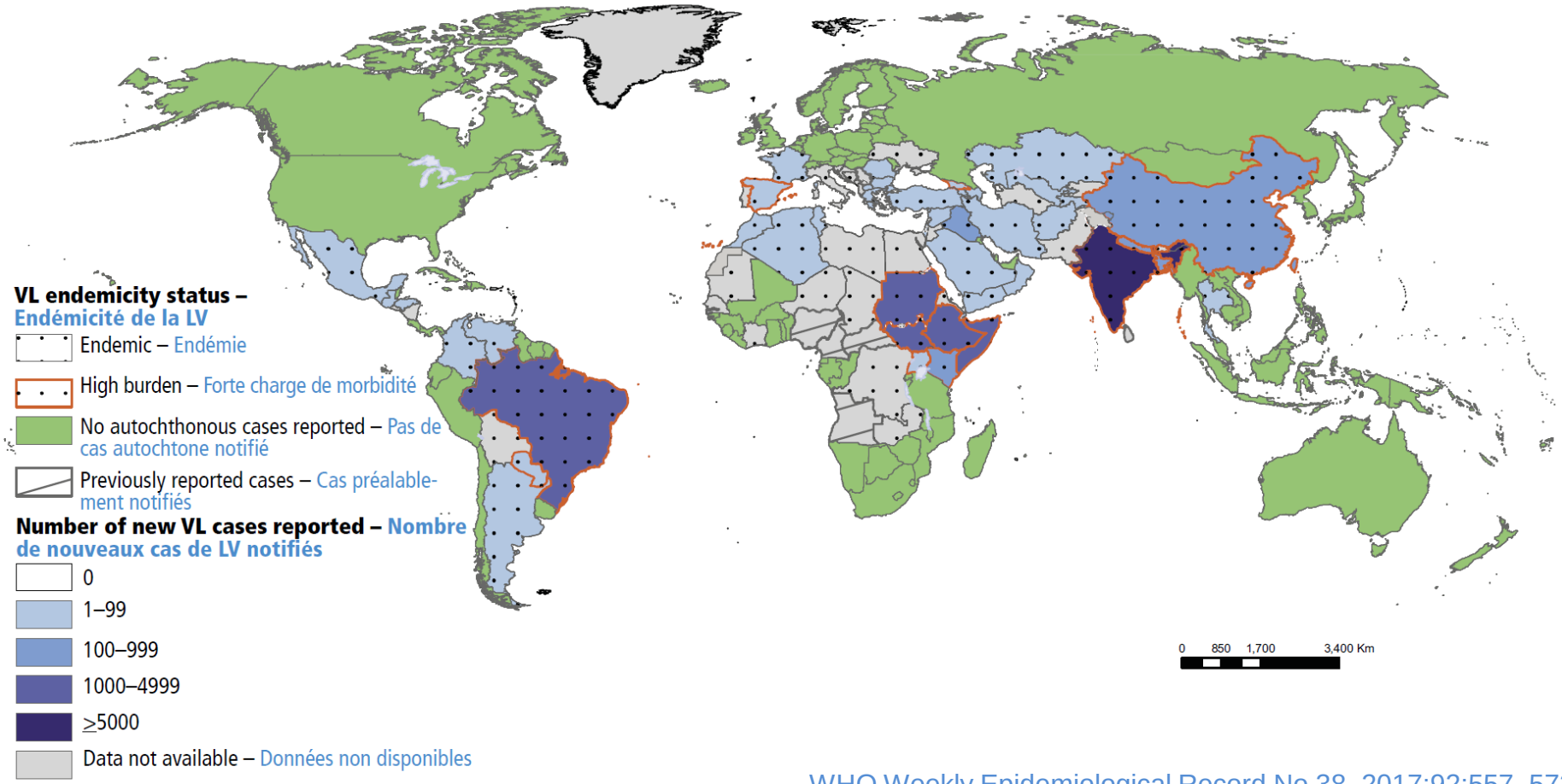


* CL: cutaneous leishmaniasis; ** VL: visceral leishmaniasis.

WHO regions: AFR: African Region, AMR: Region of the Americas, EMR: Eastern Mediterranean Region, EUR: European Region, SEAR: South-East Asia Region, WPR: Western Paci ic Region.

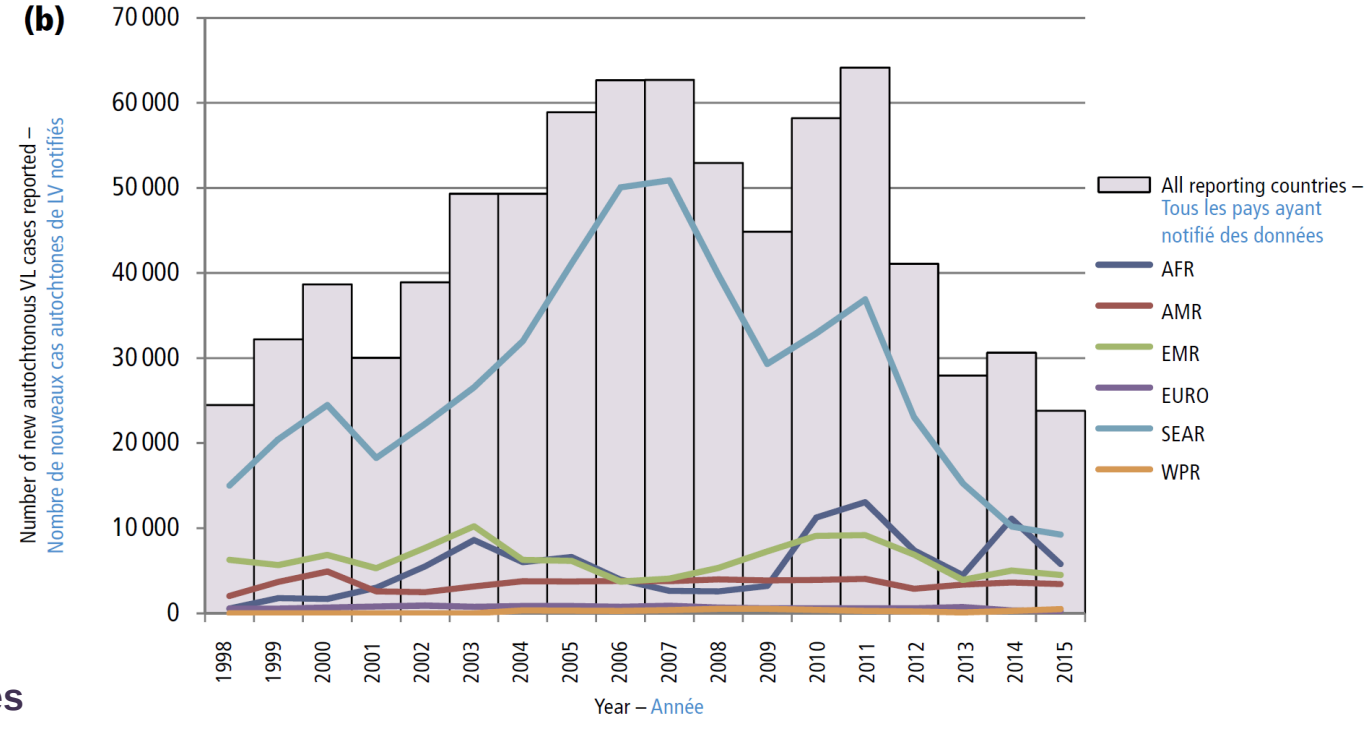
WHO Weekly Epidemiological Record No 38, 2017;92:557–572

Geographical distribution of new visceral leishmaniasis cases in 2015



WHO Weekly Epidemiological Record No 38, 2017;92:557–572

Evolution of the number of VL cases over time, by WHO region, 1998–2015



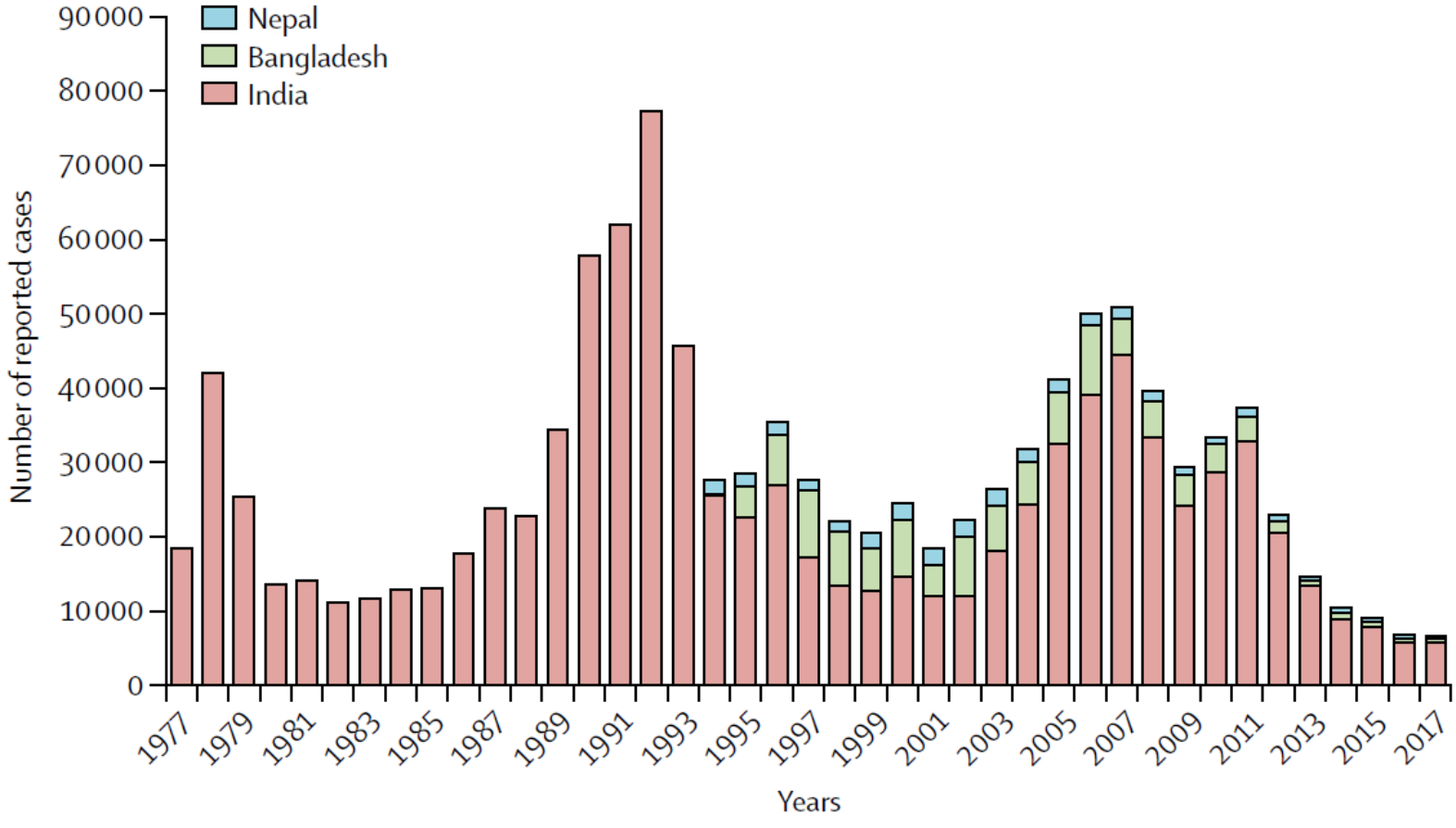
Reported cases

* CL: cutaneous leishmaniasis; ** VL: visceral leishmaniasis. – LC: leishmaniose cutanée; ** LV: leishmaniose viscérale

WHO regions: AFR: African Region, AMR: Region of the Americas, EMR: Eastern Mediterranean Region, EUR: European Region, SEAR: South-East Asia Region, WPR: Western Pacific Region. – Régions de l'OMS: AFR: Région africaine, AMR: Région des Amériques, EMR: Région de la Méditerranée orientale, EUR: Région européenne, SEAR: Région de l'Asie du Sud-Est, WPR: Région du Pacifique occidental.

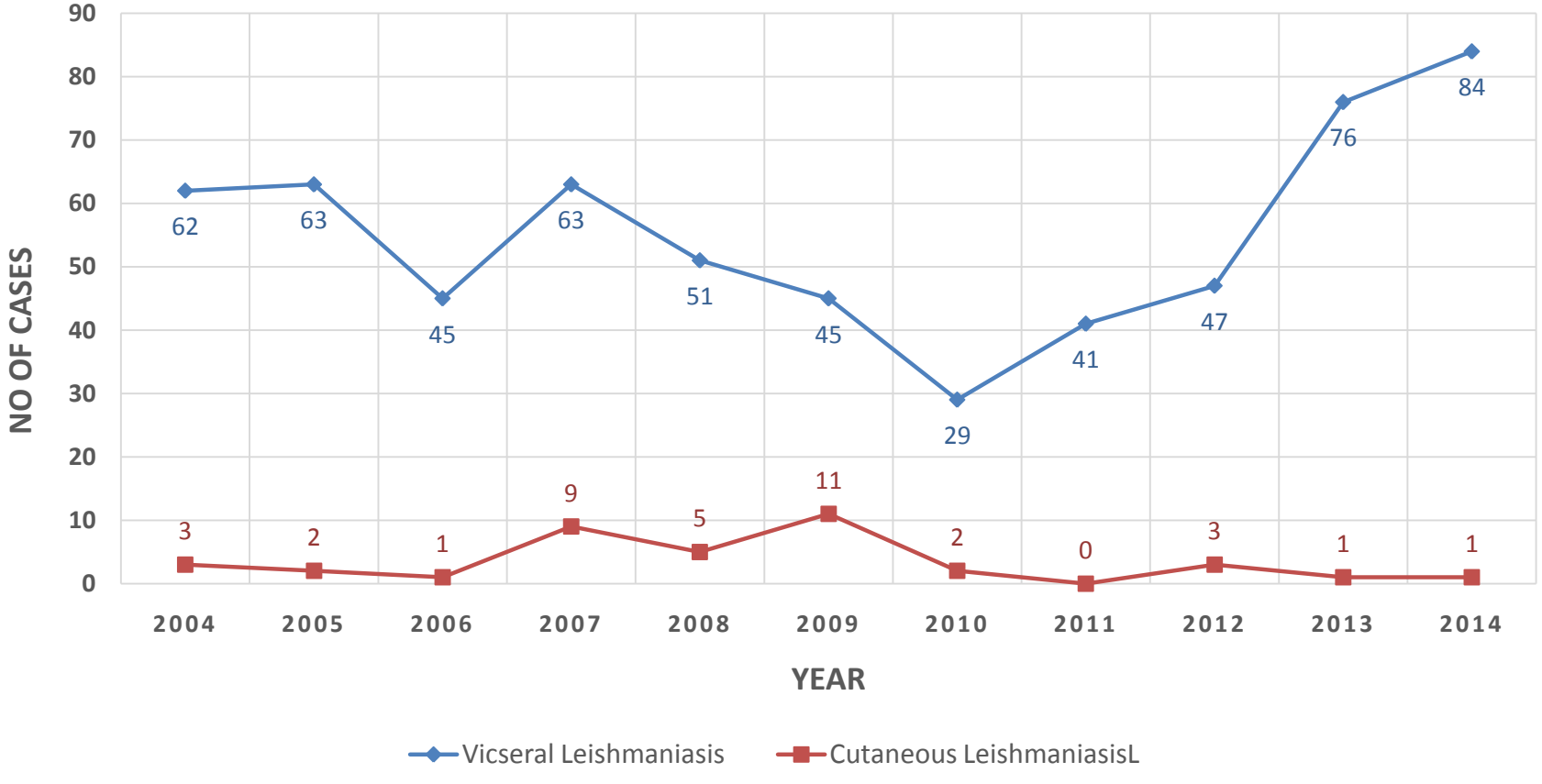
WHO Weekly Epidemiological Record No 38, 2017;92:557–572

Cyclical epidemiological patterns of visceral leishmaniasis in south Asia



Lancet 2018; 392: 951–70

Leishmaniasis in Greece

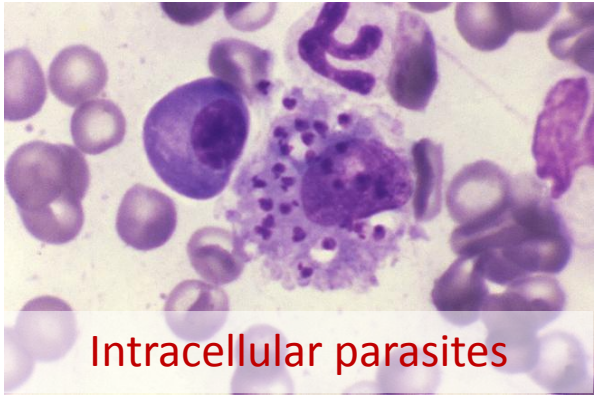


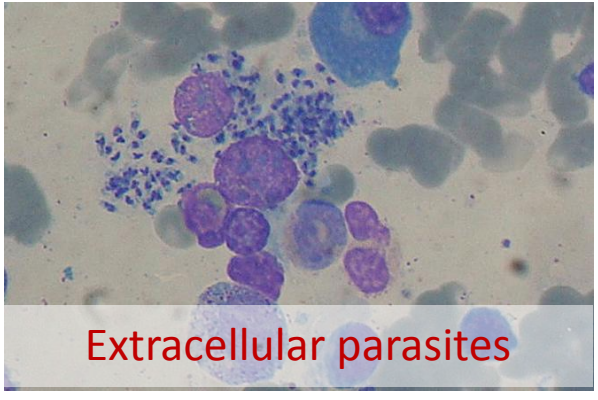
KEELPNO Bulletin 2015;48

Diagnosis of leishmaniasis

Microscopy

- Microscopy: observation of the amastigote parasite stage in tissue specimens or cultures, usually Giemsa stained.
- Amastigotes are round or oval bodies, 1–4 μm in diameter, with a typical rod-shaped kinetoplast and circular nucleus
- Gold standard: Splenic aspirates (~95% sensitivity)
  - Risk of potentially fatal haemorrhage in one per 1000 procedures
- Most countries use bone marrow, lymph node or liver aspirates:
  - IDSA 2016 Guidelines: Bone marrow is the preferred first source of specimens
  - The sensitivity of bone marrow examination was found to be proportional to the amount of time spent examining the smear: 5 min →66%, 1 hr → 92%


Intracellular parasites


Extracellular parasites

Control of the Leishmaniases. WHO 2010, Am J Trop Med Hyg. 2005;72:811.

Serological assays

- In areas with access to advanced laboratory techniques, serologic testing is used primarily for patients with suspected VL who have negative or inconclusive results for histopathology, culture, and molecular testing.
- In endemic regions with limited laboratory access, serological tests are used as the primary test to confirm the diagnosis in patients with high clinical suspicion of VL
- The sensitivity and specificity vary depending on the antigen and format used.
  - Assays that use whole parasite antigens have high sensitivity but relatively low specificity because of cross-reaction with Chagas disease, malaria, and other infections (as well as nonspecific cross-reactivity).

Serological assays

- Direct Agglutination Test (DAT): extensively validated in endemic areas and is recommended by the WHO for VL control programs.
  - Requires relatively specific material (frieze-dried antigen)
  - Can be read by eye but requires expertise in reading.
- Recombinant kinesin antigen (rK39) assays:
  - ELISA: High sensitivity in immunocompetent patients although it varies depending on the area. High specificity independent of the area.
  - Relatively limited utility in immunocompromised patients (eg HIV)
  - Rapid test (immunochromatographic strip): requires minimal equipment and is easy to use in developing settings.
  - rK39 RDT test very useful in highly endemic areas, but less so in situations of low infection intensity.
  - It cannot be used to diagnose relapses or to assess response to treatment (test of cure)
  - Approximately 10–20% of healthy people living in endemic areas test positive with the rK39 RDT

UpToDate, Clin Diagn Lab Immunol. 2002;9:951–8

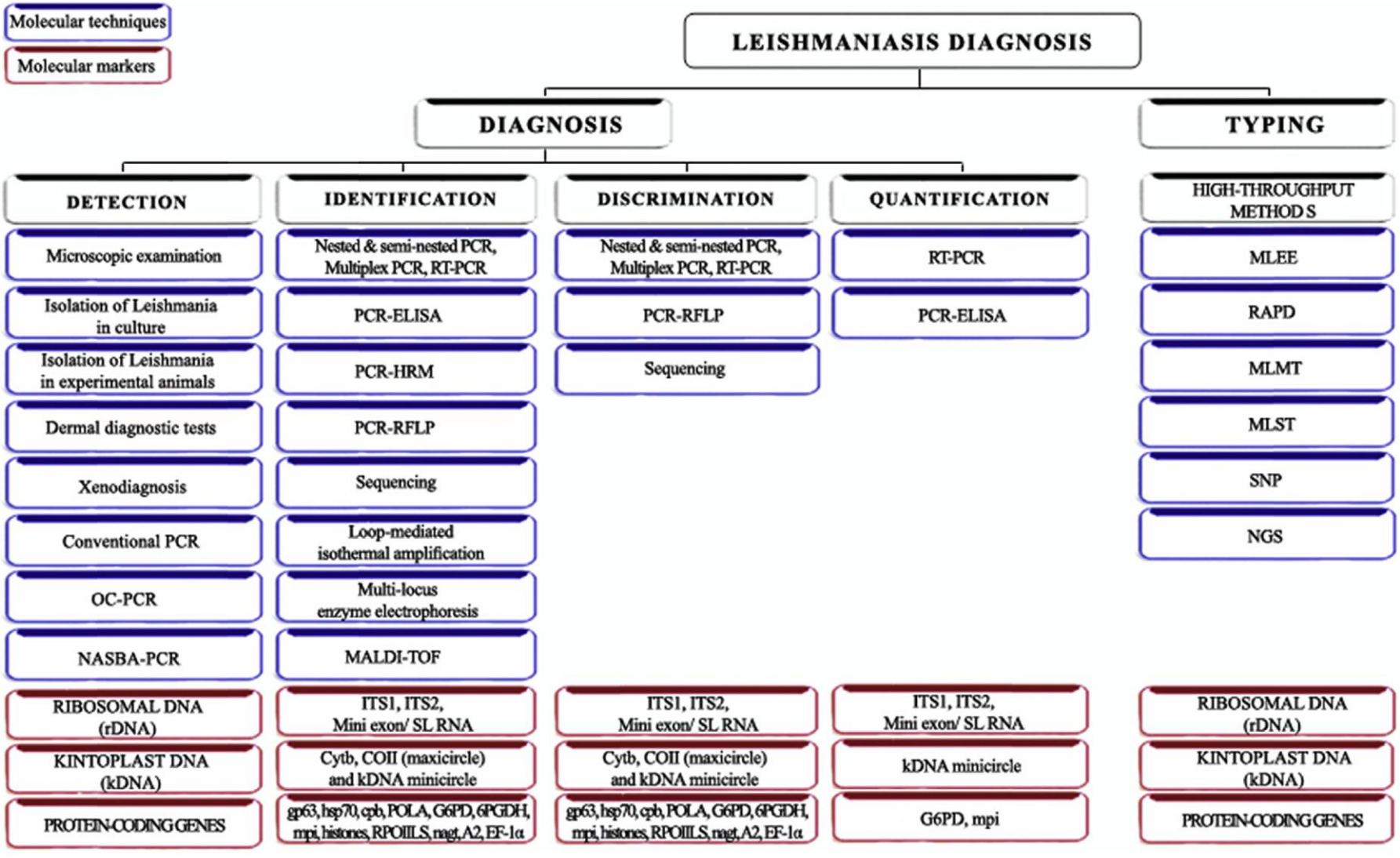
Other assays

- Culture:
  - Special parasitic growth media.
  - Growth usually occurs within two weeks but may take longer using material with few parasites.
  - The sensitivity of culture depends on the parasite load in the sampled material but is generally 60 to 85 percent
- Antigen detection: latex agglutination test (KAtex) detects the leishmanial antigen in urine.
  - Results correlate well with the parasite load.
  - Relatively low sensitivity when tested at different centers.
- Skin testing (Montenegro skin test)
  - Negative in active VL - No role in VL diagnosis.
  - Positive response 2 to 24 months after clinical recovery
  - Valuable epidemiological tool: assessment of exposure and immunity in a population.

Leishmaniasis – Diagnostic methods

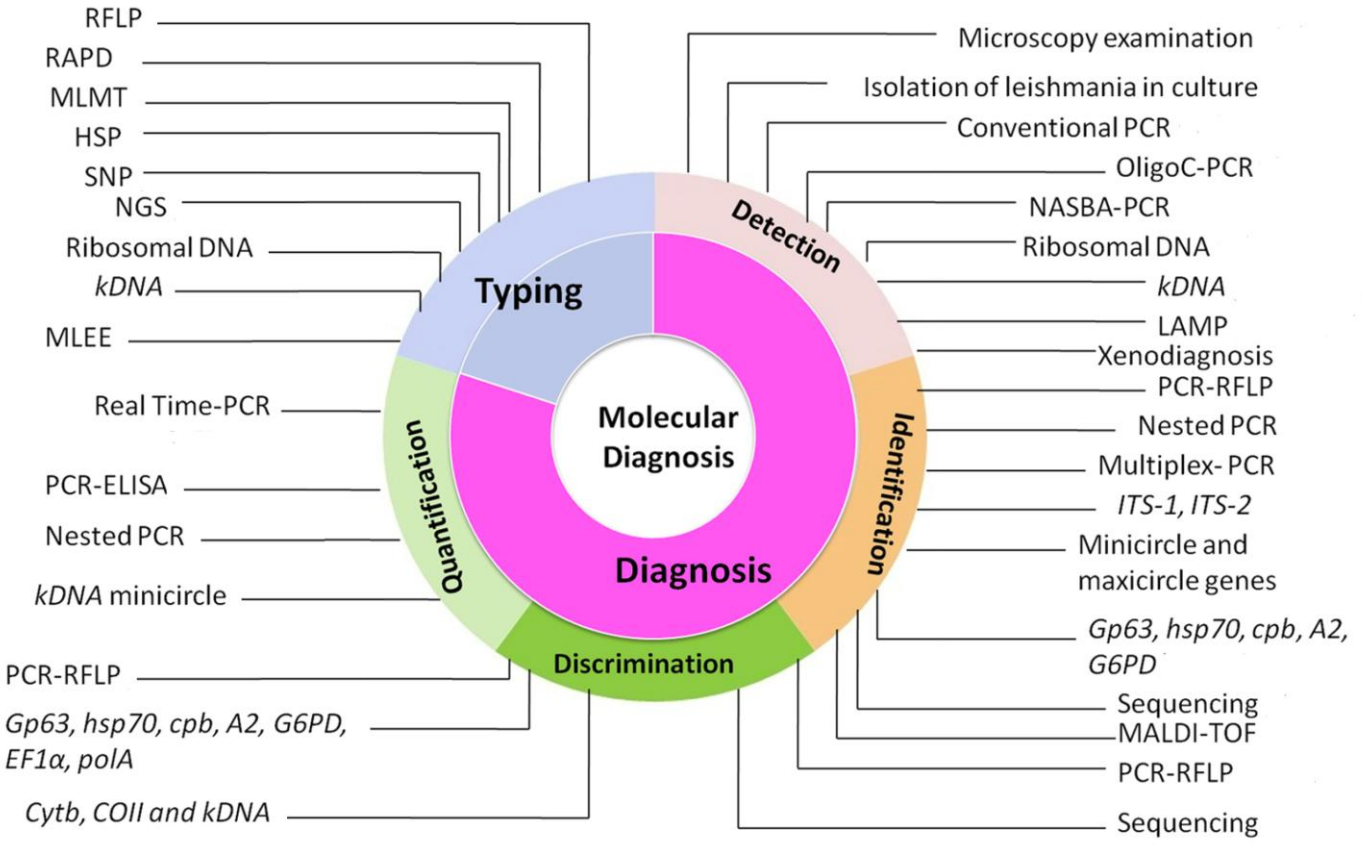
Method	Sensitivity	Specificity
Microscopy		
Spleen	93-99	100
Bone marrow	52-85	100
Lymph node	52-58	100
Antibody measurement		
IFAT	60-75	85-90
DAT	91-100	72-92
BLOT	80-100	84-95
ELISA with recombinant K39	100	100
Rapid strip test with rK39 (India)	100	88-98
Rapid strip test with rK39 (Sudan)	67-71	97-100
Antigen detection		
KATEX (India)	85-100	96-100
KATEX (Southern Europe)	69-100	92-100
PCR parasite DNA detection		
Blood	96	98
Bone marrow	90-95	96
Skin	94	98

Molecular diagnosis of leishmaniasis



Molecular Aspects of Medicine 2017;57:1-29

Laboratory tools and markers for diagnosis and typing of leishmania



Molecular Diagnosis & Therapy DOI: 10.1007/s40291-018-0343-y

Uses of molecular methods

Detection	• Detection of the presence of parasite in a clinical sample
Quantification	• Measurement of the parasite DNA load
Identification	• Establishment of the species complex of the parasite
Discrimination	• Establishment of the species of the parasite
Typing	• Phylogenetic analysis of the parasite

PCR

- Polymerase chain reaction (PCR) kits: one of the most sensitive and specific methods for diagnosis of clinical VL.
- The sensitivity of the PCR assay mostly depends on the biological sample (e.g., blood, bone marrow, splenic fluids, etc.) and the primers used to amplify the target sequence (variable or conserved target region)
- The most commonly used amplification targets are nuclear DNA such as the small subunit ribosomal RNA (SSU rRNA) gene, extra-chromosomal DNA such as repetitive kinetoplastid DNA (kDNA), mini-exon genes, and the ribosomal internal transcribed spacer (ITS) region
- One of the major limitation of DNA based PCR is the counting of dead parasite DNA
  - RNA based amplification target is preferred.

PLoS ONE. 2011;6(4):e19304, J Clin Microbiol. 2007;45:21–5.

PCR

- Increased capacity of PCR tests to detect infection in healthy individuals.
  - Leishmanial DNA has been detected by PCR in the peripheral blood of persons with asymptomatic infection in Brazil and this was also documented recently in India and Nepal.
- PCR: very useful in HIV–VL patients in whom the clinical picture is confusing and serological as well as immunological tests are not reliable due to low sensitivity.
- PCR assays have also been performed using non-invasive samples, such as buccal swabs and urine, with sensitivity of 79–83 and 88–97%, respectively
- Sensitivity and specificity of PCR can be improved significantly by performing nested and seminested PCR using two sets of primers (targeting a single gene locus) used in two successive runs.
  - The sensitivity and specificity of nested PCR using SSU-rRNA in the diagnosis of VL are reported to be 97% and 100%, respectively.

Trans R Soc Trop Med Hyg. 2002;96:S185, Am J Trop Med Hyg. 2012;86:598–600, Am J Trop Med Hyg. 2010;83:502–6.

Other PCR-based methods

- Oligochromatography-PCR (OC-PCR):
  - Simple and rapid format for detection of PCR or nucleic acid sequence based amplification (NASBA) products
  - Result is visualized on a dipstick by hybridization with a gold-conjugated probe. This detection format takes only 5-10 min and requires no equipment other than a water bath and a pipette.
  - High sensitivity, cannot discriminate various Leishmania species
- Loop-mediated isothermal amplification (LAMP):
  - Tool for point-of care diagnosis of VL and PKDL. It has been validated in several countries.
  - The test can be performed without the need for sophisticated equipment, → suitable for field-based diagnosis.
  - More rapid and cost effective than conventional PCR, but it is limited in utility due to false positivity.

Commercially available

Real-time (Quantitative) PCR

- RT-PCR: quantitative estimation of the amount of parasite DNA, but NOT the number of viable parasites
  - Can be performed in blood, buffy coat or oral fluids.
- Sensitivity depends on:
  - Assay design (primer and target region),
  - Nature of clinical samples (blood, skin, bone marrow, or splenic fluids),
  - DNA extraction methods (manual vs. commercial kits)
  - The *18S* marker is associated with the highest sensitivity and specificity
- RT-PCR can be used as a marker of treatment response: blood parasitemia in VL patients declines within a few days of drug therapy
- RT-PCR reveals difference in parasite load between primary VL and relapsed VL
- RT-PCR-positive patients have higher risk for progression of disease (odds ratio 14.8, 95% CI 5.1–42.5)

PLoS Negl Trop Dis.2014;8(12):e3366, J Antimicrob Chemother. 2011;66(8):1751–5, PLoS One. 2017;12(9):e0185606

RT-PCR

- Parasitaemia threshold for clinical disease: >5 Leishmania parasite genome detected/mL of blood
- RT-PCR parasitic burden strongly correlates with plasma IL-10
  - Biomarker of disease severity?
- PCR-ELISA: early detection and quantification of Leismania
  - Multiple sample testing using whole blood, with a sensitivity of 87%.
  - Tedious, expensive, and less sensitive than qPCR and has been tested on a limited number of clinical samples

PLoS One. 2010;5(4):e10107

Species identification & discrimination

- Species identification assays are necessary for proper management of the control programs.
  - Cutaneous clinical manifestations overlap among different species.
- Post-kala azar dermal leishmaniasis (PKDL): chronic skin rash observed following clinical response to treatment for VL due to *L. donovani:*
  - It occurs in 5-10% of VL patients in India, but in 50-60% of VL patietns in Sudan.
  - Whole-genome sequencing: large number of chromosome copy number variations between *L. donovani* strains and other *Leishmania* species
  - Better characterization of the parasite strain (i.e., species differentiation) is needed to find whether the disease is due to reactivation of persistent parasites following clinical cure of VL or re-infection.

Lancet Infect Dis.2003;3(2):87–98, Clin Infect Dis. 2009;50(1):73–6

Leishmania species identification

- Target genes: *ITS* (non-coding spacer DNA located between the *18S rRNA* and *5.8S rRNA*), repetitive nuclear DNA sequences, *cytochrome-b* genes, mini-exon genes, *G6PD* genes, *cpb* genes, *gp63* genes, and *hsp70* genes.
- Methods for species identification:
  - Restriction fragment length polymorphisms (RFLP): use of sequencing for confirmation
  - Random Amplified Polymorphic DNA technique (RAPD): arbitrarily short primes without knowing the target sequences.
  - Amplified Fragment Length Polymorphism (AFLP): advanced assay for investigation of variations in strains or closely related species
  - Multilocus sequence typing (MLST): PCR-fingerprinting

Comparison of molecular methods for detection, differentiation, identification, and quantification of

Molecular methods	Capacity to detect *Leishmania* parasites in clinical samples	Levels of *Leishmania* discrimination			Sample used	Sensitivity (%)	Specificity (%)
		G	SG	S			
PCR methods	Yes	Yes	Yes	Yes	Whole blood	70–100	85–99
					Buccal swab	79–83.00	86–90.56
					Urine	88.0–96.8	100
					Bone marrow	95.30–97	92.60–100
					Buffy coat cells	80–100	63–100
					Serum	85–96	100
					Blood-spotted filter	60–98	100
					Bone marrow-spotted filter	99–100	9.0–87
PCR-ELISA	Yes	Yes	Yes	Yes	Whole blood	83.90–100	100
Real-time PCR/qPCR	Yes	No	No	No	Whole blood	90–100	83.3–100
					Buffy coat cells	100	90
					Oral fluids	95	90–100
Oligo-C test	Yes	Yes	Yes	Yes	Whole blood	96.2	90.0
					Lymph node	65–96.8	70–100
					Bone marrow	89–96.9	57–99
Gene sequencing	Yes	Yes	Yes	Yes	Buffy coat cells	69	100
NASBA	Yes	No	No	No	Whole blood	79.8–93.3	100
					Bone marrow	85–99	50–98.9
					Lymph node	64–95	70–100
LAMP	Yes	No	No	No	Whole blood	83.0–96.4	98.0–99
					Buffy coat cells	90.7–95.0	86–100
PCR-HRM	Yes	Yes	Yes	Yes	Biopsy	NA	NA

Conclusions

- Molecular methods emerge as highly sensitive and specific tools for Leishmania parasite identification
- Conventional PCR-based methods can be used for parasite detection
- RT-PCR might be useful for assessing parasite load, evaluate response to therapy and provide prognostic information for disease progression in seropositive asymptomatic individuals
- More advanced molecular techniques will be probably used for species discrimination in the context of leismaniasis control programs and for research into pathogenetic mechanisms and for phylogenetic studies

Thank you