



GRUPPO DI STUDIO SULLA
LEISHMANIOSI CANINA

New Insights into Diagnosing Leishmaniasis

Eric Zini

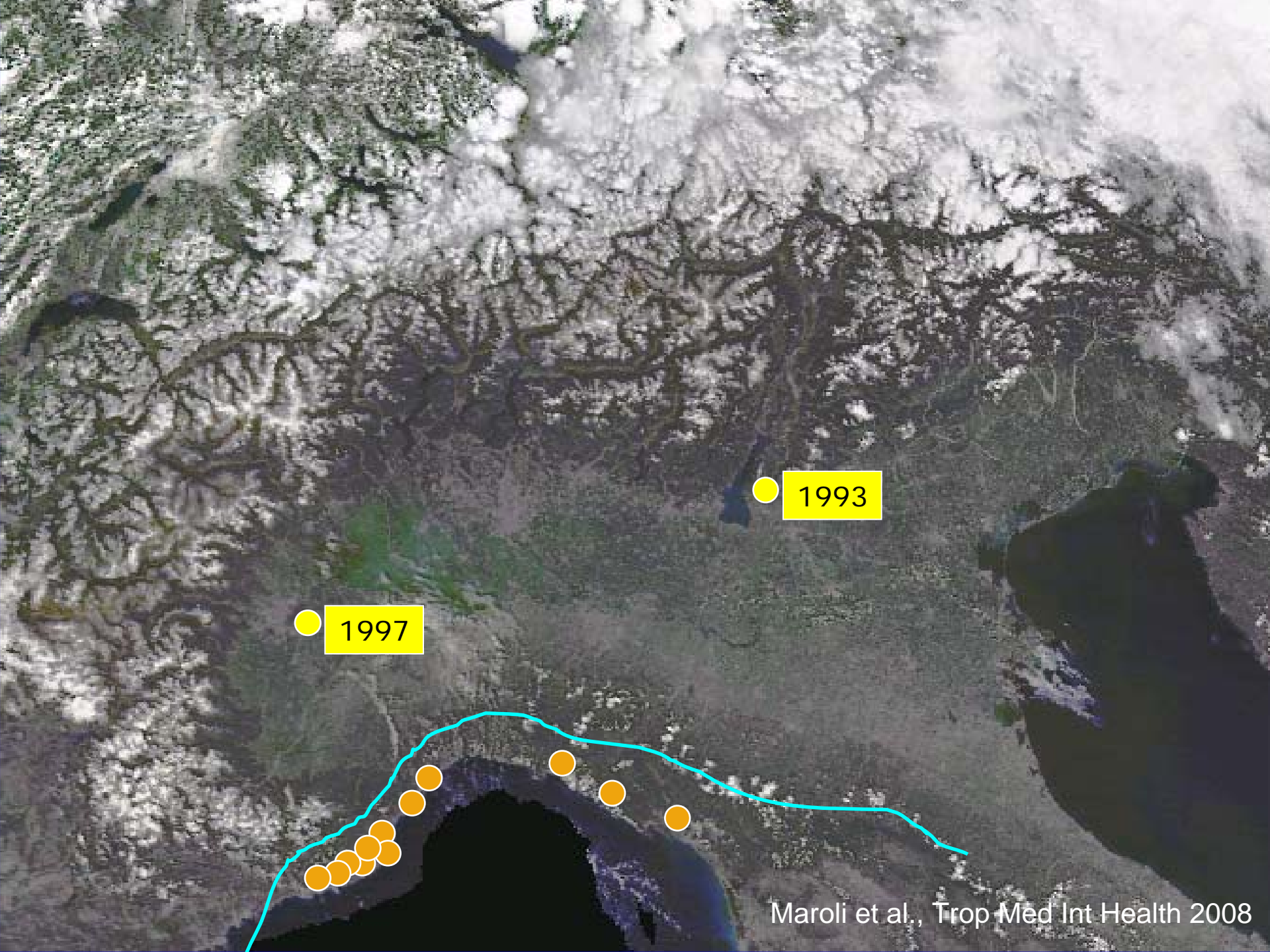
Snow meeting, 13 March 2009



Climate Variability and Visceral Leishmaniasis in Europe
 WHO/TDR, Jan. 2008

Late Eighties

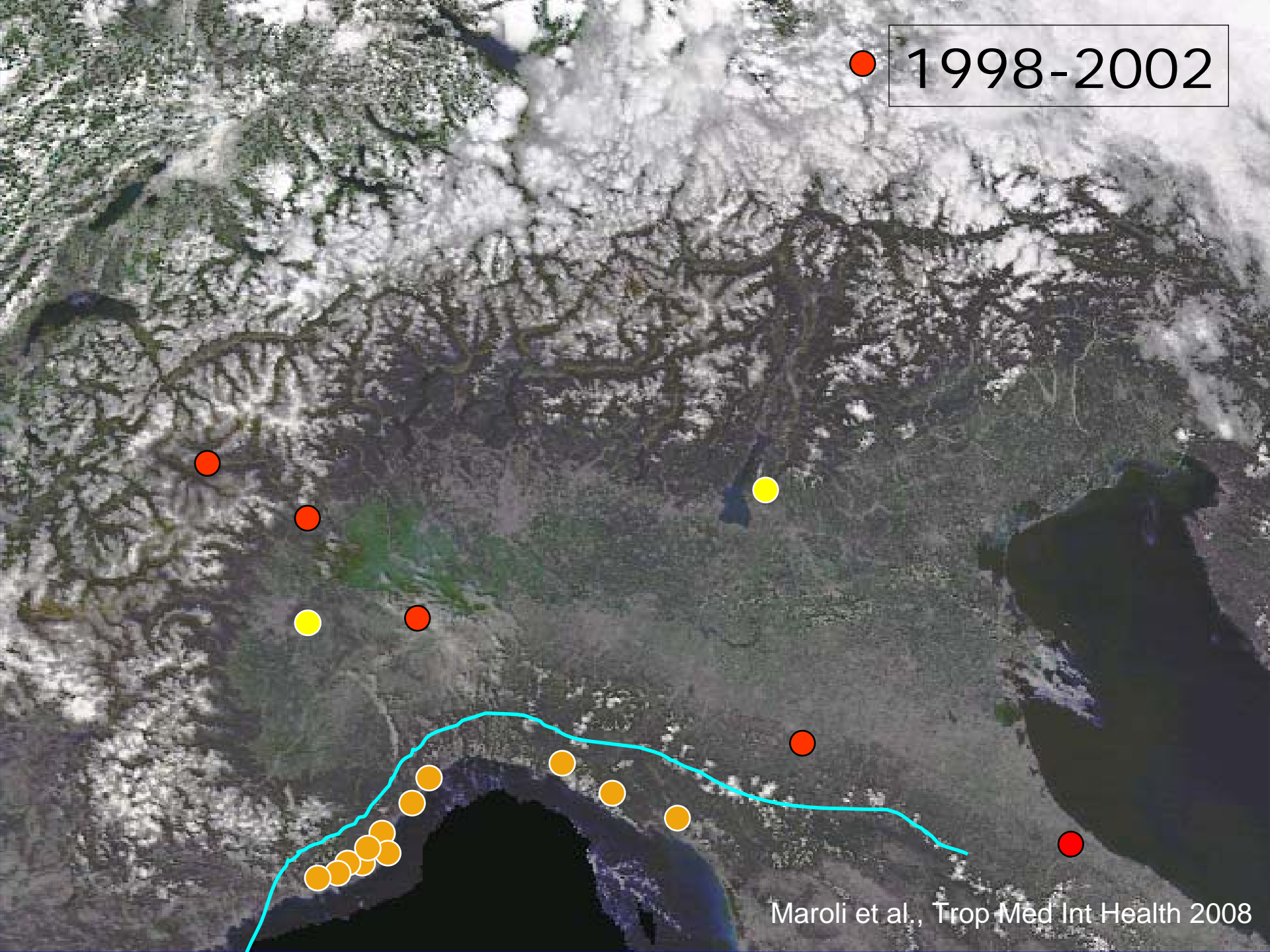




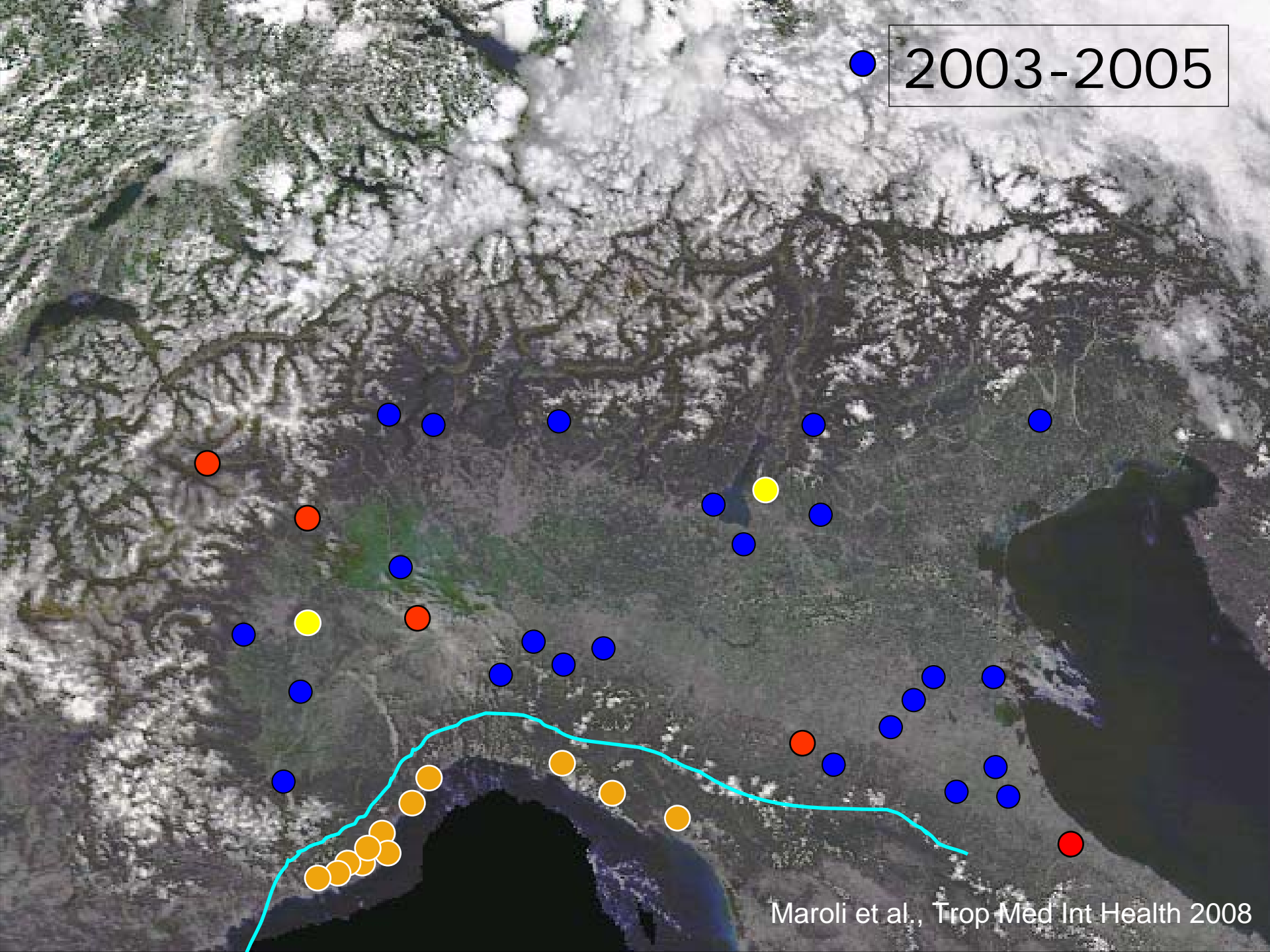
1993

1997

● 1998-2002



● 2003-2005



Remark

- ✓ Increased incidence in endemic zones
- ✓ Northward spread to non-endemic areas in UE
- ✓ Emergence in North America
- ✓ Approach to disease very heterogenous



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Remark

- ✓ Increased incidence in endemic zones
- ✓ Northward spread to non-endemic areas in UE
- ✓ Emergence in North America
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**consensus for diagnosis, therapy
and prevention is missing**



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Canine Leishmaniasis Working Group



Canine Leishmaniasis Working Group

- | | | |
|------------|--|---------------------------|
| 1. | Prof. MASSIMO CASTAGNARO, DECVP | Histopathology |
| 2. | Dr. ALBERTO CROTTI | Ophthalmology |
| 3. | Dr. ALESSANDRA FONDATI, DECVD | Dermatology |
| 4. | Dr. LUIGI GRADONI, BioSc | Parassitology |
| 5. | Prof. GEORGE LUBAS, DECVIM-CA | Hematology |
| 6. | Dr. MICHELE MAROLI, BioSc | Entomology |
| 7. | Prof. GAETANO OLIVA | Internal Medicine |
| 8. | Prof. SAVERIO PALTRINIERI, DECVCP | Clinical pathology |
| 9. | Dr. XAVIER ROURA, DECVIM-CA | Internal Medicine |
| 10. | Dr. LAIA SOLANO-GALLEGO, DECVCP | Immunology |
| 11. | Dr. ANDREA ZATELLI | Chairman |
| 12. | Dr. ERIC ZINI, DECVIM-CA | Nephrology |



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Canine Leishmaniasis Working Group

- ✓ AIM: to provide a scientific-based consensus for the management of canine leishmaniasis regarding diagnosis, clinical classification, therapy and prevention
- ✓ M&Ms: review of international literature and, if incomplete, supplemented with personal experience
- ✓ In collaboration with the “Dept. of Health and Epidemiology, Section Guidelines” (Modena, Italy)

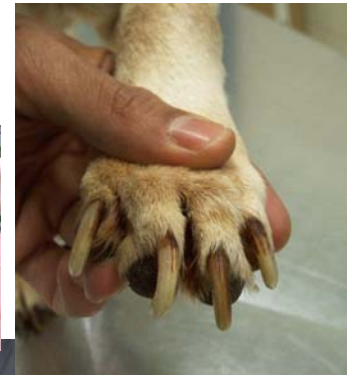
Suspect of leishmaniasis

- ✓ Signalment and History
 - Predisposed breeds: German shepherd, Boxer
 - Predisposed gender: male
 - Bimodal age distribution: < 3 and 8-10 years
 - Living/travelling to endemic areas
 - Prevention
 - Drugs interfering with immune response

Suspect of leishmaniasis

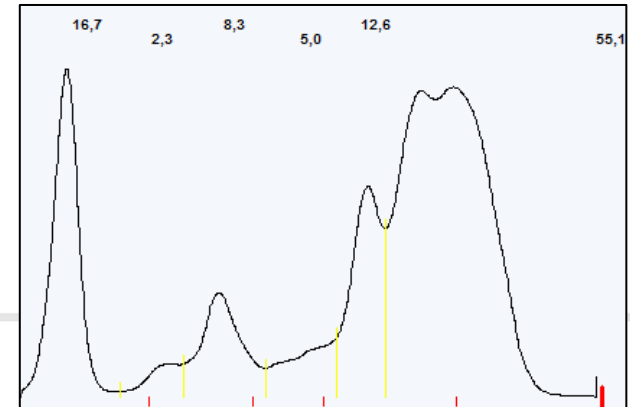
✓ Physical examination

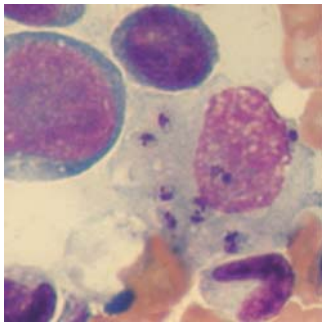
- General
- Cutaneous and mucocutaneous
- Ocular
- Others



✓ Basic laboratory tests

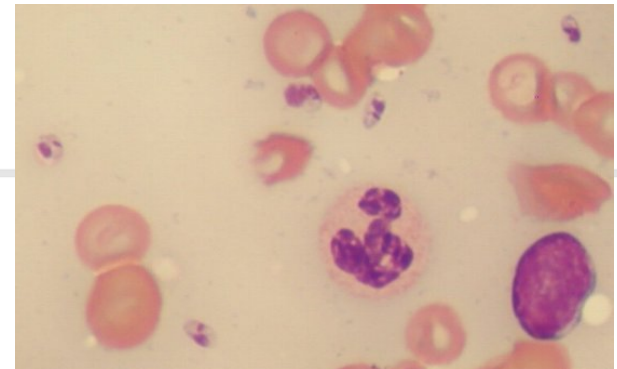
- Complete blood count
- Serum biochemical panel
- Serum protein electrophoresis
- Urinalysis





Direct diagnostic methods: CYTOLOGY

- ✓ Demonstration of amastigotes in macrophages
- ✓ Extracellular parasites if heavy infection
- ✓ Changes consistent with leishmaniasis
 - Lymphoplasmacytic inflammation
 - (Pyo-)granulomatous inflammation
 - Lymph node reactive hyperplasia
 - Myeloid hyperplasia, erythroid hypoplasia



Direct diagnostic methods: CYTOLOGY

- ✓ Good evidence for cause-effect relationship if fine needle aspiration biopsy from:
 - Papular, nodular, ulcerative* skin lesions
(* *also impression smears*)
 - Bone marrow and lymph nodes when clinical signs
 - Biological fluids from affected sites (e.g., synovial fluid, CSF)

Direct diagnostic methods: CYTOLOGY

- ✓ If organ/tissue related clinical signs are absent, samples from sites in which parasites more likely:
 - Bone marrow
 - Lymph node
 - Spleen
 - Buffy-coat
- } ↓ diagnostic sensitivity
- ✓ In case of negative findings, stored aliquot processed for molecular diagnosis (e.g., PCR)

Mylonakis et al., Vet Clin Pathol 2005

Direct diagnostic methods: HISTOLOGY

- ✓ Parasites can be demonstrated in tissue sections stained with H&E, but identification is challenging
- ✓ Compatible histopathologic changes
 - Same as for cytology (...)
 - Vasculitis
 - Lymphoplasmacytic dermatitis of dermoepithelial junction



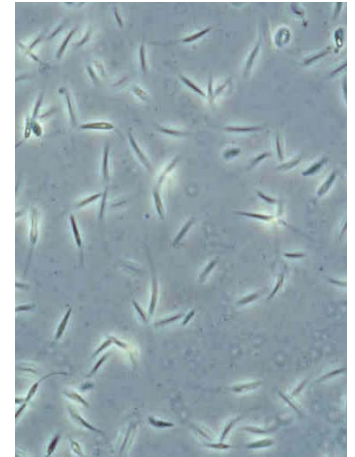
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Direct diagnostic methods: HISTOLOGY

- ✓ Histology advisable if leishmaniasis is suspected but cytology is negative and focal skin lesions are observed
- ✓ If histopathologic changes but no parasites are detected:
 - Immunohistochemistry against *Leishmania* antigen
 - If negative, tissue processed for molecular diagnosis (e.g., PCR)

Roura et al., J Vet Diagn Invest 1999

Direct diagnostic methods: CULTURE



- ✓ Specific assay but low sensitivity
- ✓ Media are not commercially available (e.g., RPMI, Schneider's *Drosophila* medium)
- ✓ Growth requires up to 30 days

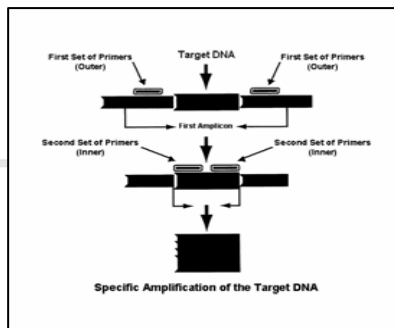
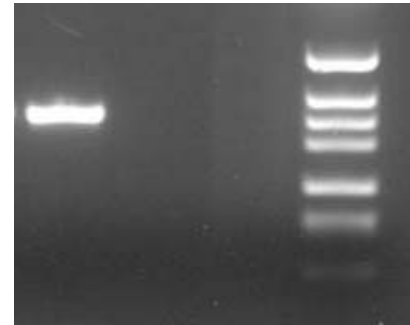
Gradoni et al., OIE Office International des Epizooties, 4th ed., 2000

Direct diagnostic methods: PCR

- ✓ Detection of *Leishmania* genome sequences
- ✓ Very sensitive, if multicopy DNA is targeted (e.g., rRNA genes, kinetoplast DNA minicircles)

- ✓ Techniques:

- Conventional PCR →
- Nested PCR

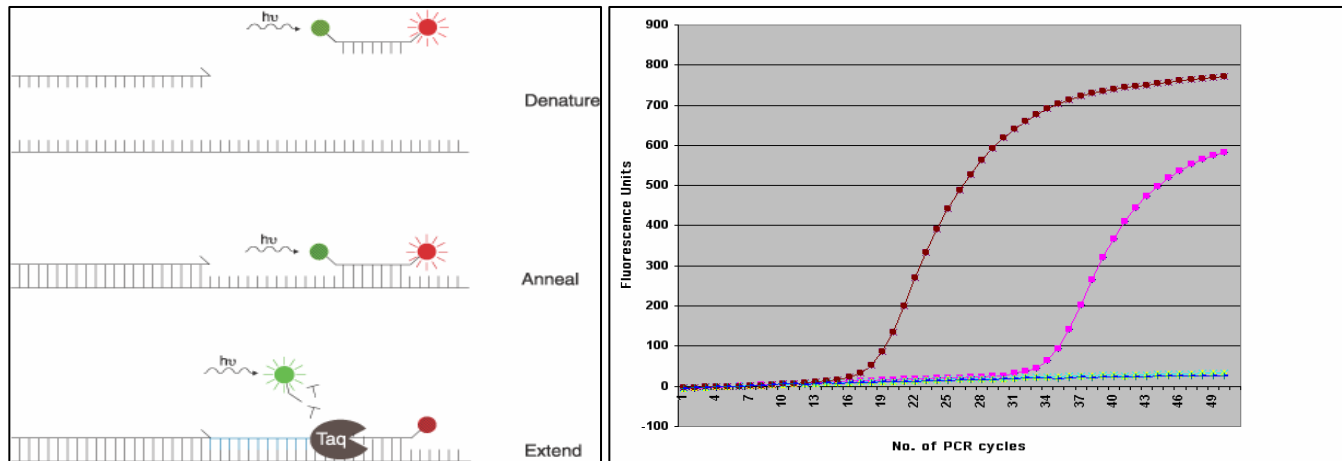


Francino et al., Vet Parasitol 2006

Direct diagnostic methods: PCR

✓ Techniques:

- **Realtime PCR**
 - To monitor efficacy of therapy
 - First-line diagnostic approach



Francino et al., Vet Parasitol 2006

Direct diagnostic methods: PCR

- ✓ Applied to several biologic samples
- ✓ In addition to injured tissues, samples with highest chances are:

- Bone marrow / lymph nodes
- Skin
- Conjunctiva (sens. 92%)
- Buffy coat
- Whole blood

↓ diagnostic sensitivity

Maia and Campino, Vet Parasitol (*in press*)

Direct diagnostic methods: PCR

IMPORTANT

In dogs with efficient immune responses against *Leishmania* infection may not progress further:

1. In endemic area, PCR positive skin without skin lesions may not suggest established infection or disease development

Oliva et al., J Clin Microbiol 2006

Direct diagnostic methods: PCR

IMPORTANT

In dogs with efficient immune responses against *Leishmania* infection may not progress further:

1. In endemic area, PCR positive skin without skin lesions may not suggest established infection or disease development
2. In endemic area, PCR positive bone marrow during or shortly after exposure may be followed by negativization

Oliva et al., J Clin Microbiol 2006

Direct diagnostic methods: XENODIAGNOSIS

- ✓ Laboratory-bred sandflies on the suspected dog
- ✓ Vectors are examined after blood digestion for promastigotes
- ✓ Very efficient “culture medium”
- ✓ Not widely applicable

Gradoni et al., OIE Office International des Epizooties, 4th ed., 2000

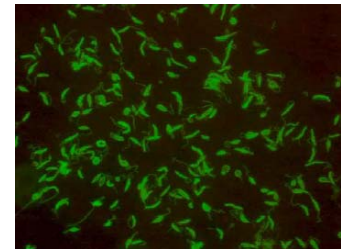
Indirect diagnostic methods: SEROLOGICAL METHODS

- ✓ Seroconversion:
 - in natural infection, 5 months (range: 1-22)
 - in experimental infection, 3 months (range: 1-6)
- ✓ ↑titers or increasing overtime if parasite dissemination
- ✓ Serological methods:
 - Indirect immunofluorescent assay test (IFAT)
 - ELISA
 - Rapid immunochromatographic strip test

Moreno et al., Trends Parasitol 2002

Indirect diagnostic methods: IFAT

- ✓ Serial serum dilutions on *Leishmania*-coated slides
- ✓ Titer revealed by fluorescent anti-antibodies
- ✓ Subjective
- ✓ High sensitivity and specificity



recommended as reference by the
World Organization for Animal Health (OIE)

Mettler et al., J Clin Microbiol 2005

Indirect diagnostic methods: IFAT

- ✓ Positive titer varies widely (1/40 to 1/320)
- ✓ Use same reference lab
- ✓ Proposal
 - “high-titer” if > 2-4-fold higher than the threshold
 - “low-titer” if 1-2-fold higher than the threshold
- ✓ Cross-reactivity with other infections

Kjos et al., Vet Parasitol 2008

Indirect diagnostic methods: ELISA

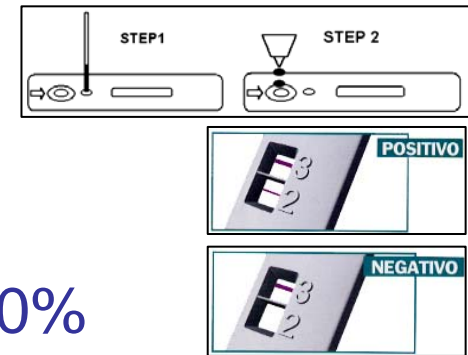


- ✓ Information on the diagnostic performance comes from manufacturers
 - ✓ Serum in *Leishmania* antigen-coated microplates
 - ✓ Colorimetric reaction and spectrophotometry
- ⇓
- no subjective evaluation
- ✓ Specific test, sensitivity according to # of antigens

Reithinger et al., J Clin Microbiol 2002

Indirect diagnostic methods: RAPID IMMUNOCHROMATOGRAPHIC STRIP TEST

- ✓ Several kits, different antigens and reagents
- ✓ Easy-to-use
- ✓ ↓ performance than ELISA or IFAT
- ✓ Medium-high specificity, sensitivity 30-70%
- ✓ Does not indicate antibody titer



⇓
false negatives

Mettler et al., J Clin Microbiol 2005

Indirect diagnostic methods: SUMMARY

	SENSITIVITY	SPECIFICITY
• IFAT	HIGH (>98%)	HIGH (100%)
• ELISA	HIGH (>95%)	HIGH (100%)
• Immunomigration	LOW (30-70%)	MIDDLE (50-70%)

Mettler et al., J Clin Microbiol 2005
Reithinger et al., J Clin Microbiol 2002





Integration of diagnostic tests

- ✓ In general, if overt clinical signs and/or severe laboratory alterations, first-line diagnosis with cytology of injured tissues or serology (or PCR)
- ✓ More difficult in endemic areas, if vague signs



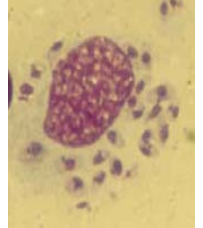
Cause-effect relationship?

...risk to overestimating leishmaniasis

Leontides et al., Vet Parasitol 2002

Possible combinations

1. When injured tissues (including bone marrow if anemia) are positive by cytology, the dog is affected, regardless of serology

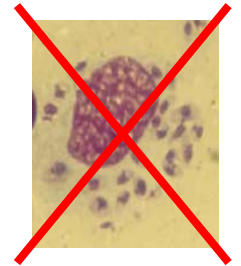


in general “high-titer”, except (rare):

- A) extremely localized lesions
- B) clinical signs anticipate antibody response

Possible combinations

2. When injured tissues are negative by cytology, serology is crucial:



A) “high-titer” confirms the disease

B) “low-titer” may only suggest *Leishmania* infection and the dog is affected by another disease sharing signs



...thus, other diagnostics according to presentation

Possible combinations

i. If cutaneous lesions (cytologically compatible), a skin biopsy to histologically identify *Leishmania*:

- If routine staining negative → immunohistochemistry
- If immunohistochemistry negative → PCR



If negative...



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Possible combinations

- ii. If non-cutaneous lesions (e.g., systemic signs):
 - PCR in tissues with the highest diagnostic chance (bone marrow or lymph node)



If negative, these “cytology-negative low-seropositive dogs” should be considered:

Possible combinations

ii. If non-cutaneous lesions (e.g., systemic signs):

- PCR in tissues with the highest diagnostic chance (bone marrow or lymph node)



If negative, these “cytology-negative low-seropositive dogs” should be considered:

➤ *Leishmania*-free (the “low-titer” due to previous exposure to parasite), with another disease

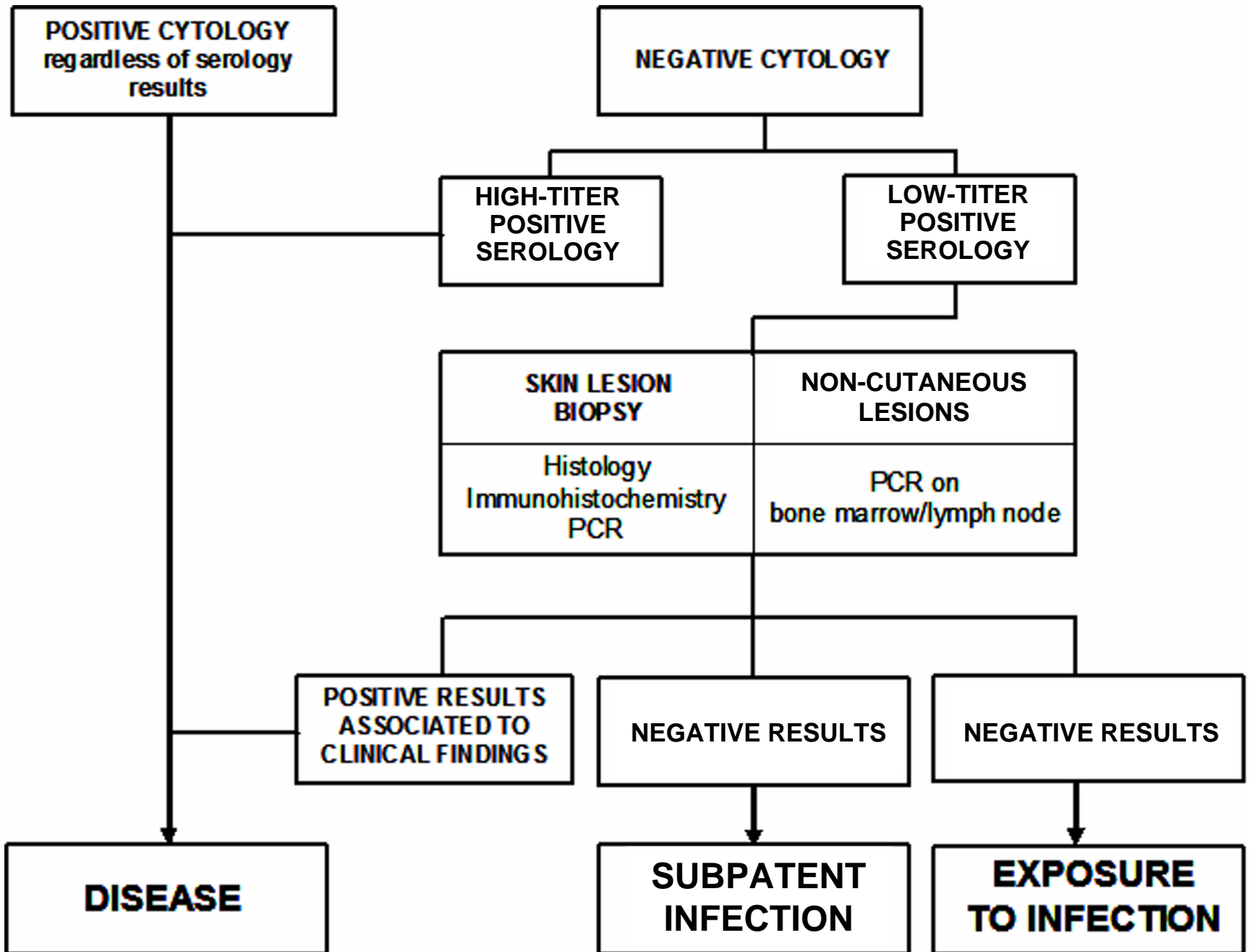
or

➤ Infected with *Leishmania* at the subpatent level, with another disease

→ monitoring recommended



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Approach to clinical classification

- ✓ Leishmaniasis evolves toward variable and polymorphic disease patterns
- ✓ To establish adequate treatment or predict progression to more serious stages



Proposal for clinical classification
of use for management of affected dogs

Approach to clinical classification

EXPOSURE or SUBPATENT INFECTION

- ✓ Negative cyto-histologic and PCR findings
- ✓ “Low-titer” positive serology
- ✓ Clinically healthy or other disease
- ✓ Dogs living or lived during one/more transmission seasons in a territory with *Leishmania* vectors



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Approach to clinical classification

PATENT INFECTION

- ✓ Parasites confirmed with direct methods
- ✓ “Low-titer” positive serology
- ✓ Clinically healthy or other disease
- ✓ In endemic areas, positive findings by skin or blood PCR in the absence of lesions and during the infection transmission period may not indicate infection (promastigote DNA)



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Approach to clinical classification

DISEASE

- ✓ Infected dogs, showing one/more clinical signs or laboratory alterations compatible with leishmaniasis
- ✓ “High-titer” (or “low-titer”) positive serology



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Approach to clinical classification

SEVERE DISEASE

- ✓ Diseased dogs with a severe clinical picture, including:
 - i. unresponsiveness to repeated drug treatments
 - ii. chronic renal failure
 - iii. concurrent problems related or not to leishmaniasis requiring immunosuppression
 - iv. severe concomitant disorders



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