
Stealth infections and their detection

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AONM Annual Conference London, November 19th 2017



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What are stealth pathogens?

- ▶ Expert at hiding from the immune system
- ▶ Disable/interfere with the cytokine system (immune system's chemical messengers)
- ▶ Chronic infection associated with chronic immune dysfunction
- ▶ Symptoms often vague and non-specific: extremely difficult to diagnose
- ▶ Highly sophisticated persistence mechanisms: state of "reversible quiescence"
- ▶ Can alter their genotype in response to changes in their environment
- ▶ Preferred location mostly intracellular; frequently try to shift the host's immune response from Th1 (intracellular) towards Th2 (extracellular) to distract attention from their location
- ▶ Initiate processes to stop infected cells from dying, so their habitat is kept alive

Stealth pathogen symbiosis

The emergence of the Lyme group of pathogenic microbes is deeply related to ecosystem disruption. **Stealth microbes are a stronger force together than when alone.** In other words, Mycoplasma (for example) may not be a problem unless other stealth microbes are present.

And no mistake: these stealth pathogens are **highly intelligent.**

Source: <http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/pulmonary/sarcoidosis/>

Multiple stealth pathogens associated with Lyme Disease

- ▶ **Mycoplasma**
- ▶ **Bartonella**
- ▶ **Babesia**
- ▶ **Ehrlichia/Anaplasma**
- ▶ **Chlamydia pneumoniae**
- ▶ **Yersinia**
- ▶ **Coxsackie viruses (B1, A7, A16)**

... & many others. The archetypal stealth pathogen is Borrelia itself, in all its forms

Agenda for today's presentation

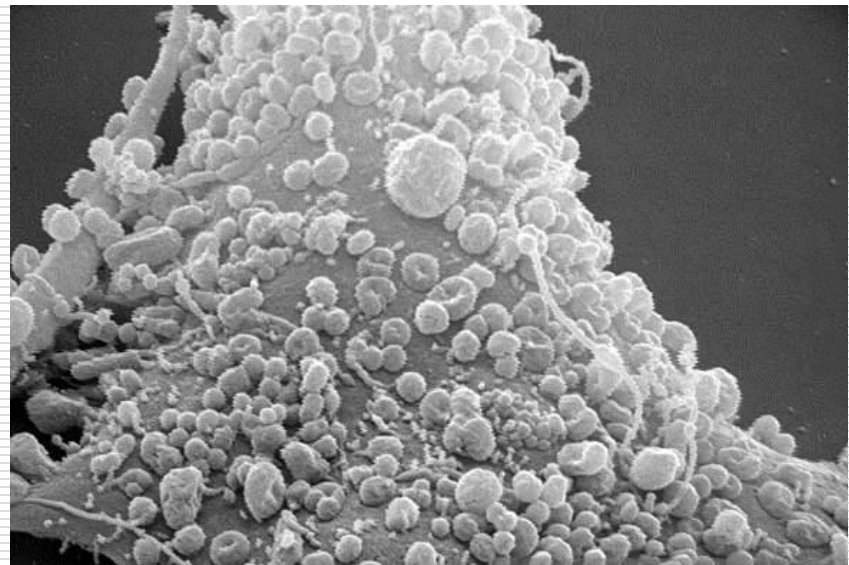
▶ Deep-dive into a few stealth pathogens commonly associated with Lyme Disease

- Mycoplasma
- Bartonella
- Babesia
- Ehrlichia/Anaplasma
- Yersinia

▶ How to test for active infection, even if chronic

Mycoplasma:

Mycoplasmas are the stealthiest of all stealth microbes. They are the smallest free-living organisms on the planet (150 – 250 nm), and lack a cell wall. No. 1 coinfection in Lyme patients – ~ 70%. As far back as 2002, Professor Nicolson found that 70% of a cohort of ME patients were infected with at least one strain of Mycoplasma. They generally prefer low-oxygen environments, and stimulate reactive oxygen species (ROS), which cause damage to cell membranes – membrane potential is lost.



Mycoplasma on the surface of a fibroblast

Source: Nicolson GL et al. Chronic Fatigue Syndrome Patients Subsequently Diagnosed with Lyme Disease *Borrelia burgdorferi*: Evidence for Mycoplasma Species Coinfections. *Journal of Chronic Fatigue Syndrome*, Volume 14, 2007 - Issue 4

Vectors/nutrition

Spread by biting insects (ticks, mosquitoes, fleas, biting flies), **contaminated food, and human to human via airborne droplets.**

Mycoplasma have lost almost all the genes required for making nucleotides, amino acids, etc., so they scavenge everything they need for survival from their host (vitamins, minerals, fats, carbohydrates, and amino acids).

To gain access to needed resources, mycoplasma generate inflammation by manipulating the signalling mechanisms of the immune system (cytokines). Inflammation breaks down tissues and allows the bacteria to gain access to the host's resources.

Source: Razin S et al. *Molecular Biology and Pathogenicity of Mycoplasmas*. [Microbiol Mol Biol Rev](#). 1998 Dec; 62(4): 1094–1156; Yang J et al. *Regulation of Proinflammatory Cytokines in Human Lung Epithelial Cells Infected with Mycoplasma pneumoniae*. [Infect Immun](#). 2002 Jul; 70(7): 3649–3655

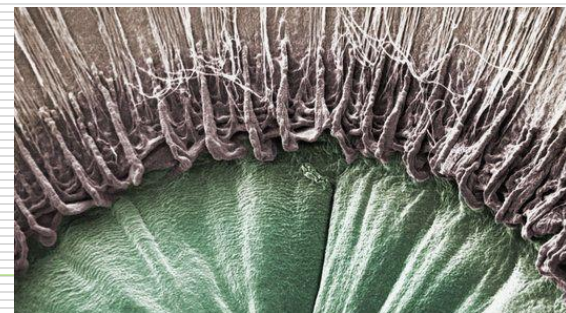
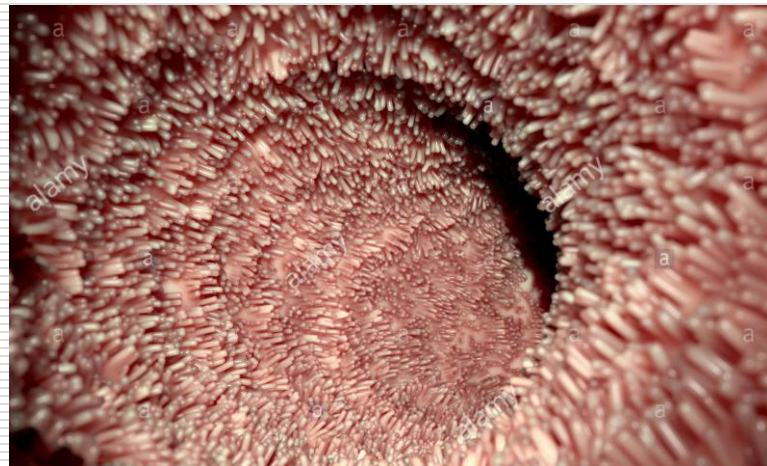
Drawn to ciliated structures

Most mycoplasma have a huge affinity for mucous membranes and ciliated cells/cellular structures. Various mucous membrane systems associated with ciliary structures:

- 1) Small intestine**
 - 2) Entire respiratory system**
 - 3) Vagina, fallopian tubes and uterus**
 - 4) Vesicles of the brain that circulate cerebrospinal fluid, cilia of the eyes' photoreceptors**
 - 5) Synovial tissues in the joints**
- ... etc.**

Most mycoplasmal symptoms come from infection and damage of cilia. Mycoplasma use inflammation to make epithelial and endothelial structures more porous, penetrating to deeper cilia, giving access even to the mitochondria.

Source: *Ciliary and Flagellar Membranes*. Bloodwood, E (Ed.), 1990, Springer; Prince OA et al. *In Vitro Spatial and Temporal Analysis of Mycoplasma pneumoniae Colonization of Human Airway Epithelium*. *Infect Immun*. 2014 Feb; 82(2): 579–586



Mycoplasma: symptoms

Pulmonary: Pneumonia, bronchitis, pharyngitis, rhinitis, earaches, sinusitis

Gastrointestinal: Hepatitis, pancreatitis

Rheumatic: Arthritis, arthralgias, myalgias, polyarthritis

Kidneys: Glomerulonephritis

Ocular: Uveitis

Neurological: Myelitis, Guillain-Barré syndrome, encephalitis, meningitis, polyradiculopathy, peripheral facial paresis optical neuritis, hemorrhagic leukoencephalitis, peripheral polyneuropathy, cranial nerve neuritis, radiculitis

Source: Berghoff W. Chronic Lyme Disease and Co-infections: Differential Diagnosis. *Open Neurol J.* 2012; 6: 158–178. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3565243/table/T3/>; Kashyap S, Sarkar, M. *Mycoplasma pneumonia: Clinical features and management. Lung India.* 2010 Apr-Jun; 27(2): 75–85.

Mycoplasma and autoimmunity

Prof. Garth L. Nicolson

Chronic Fatigue Syndrome, Fibromyalgia Syndrome and Other Fatigue Conditions

Chronic fatigue is reported by 20% of all patients seeking medical care and is considered as a nonspecific sign that is associated with many well known medical conditions. Chronic Fatigue Syndrome (CFS), Myalgic Encephalomyelitis (ME), and Fibromyalgia Syndrome (FMS) patients suffer from complex overlapping signs and symptoms. (see 'Signs/Symptoms' Questions, above) CFS is primarily characterized by persisting or relapsing fatigue without previous history of comparable symptoms that does not resolve with rest. In these patients other clinical conditions are absent that can explain the signs and symptoms such as malignancies or **autoimmune** diseases. In contrast, FMS patients have overall muscle pain, tenderness, and weakness as primary complaints, but they have most if not all of the commonly found signs and symptoms for CFS. We previously proposed that CFS/ME patients might be suffering from chronic infections that can cause, in part, their complex signs and symptoms. For example, systemic mycoplasmal infections can cause chronic fatigue, muscle pain and a variety of additional signs and symptoms, some of which are related to dysfunctional immune responses and in extreme cases **autoimmune**-like disorders. Some mycoplasmas can invade virtually every human tissue and can compromise the immune system, permitting opportunistic infections by other bacteria, viruses, fungi and yeast. When mycoplasmas exit certain cells, such as synovial cells, nerve cells, among others that can be infected, they can stimulate **autoimmune** response. Our recently published studies demonstrated a possible link between mycoplasmal infections and CFS and FMS, since we found high frequencies of mycoplasmal infections in these patients.

As Mycoplasma replicate within cells and are eventually released, they capture antigens from the surface of the host cell and incorporate these antigens into their own membranes. This makes it almost impossible for the body to tell the difference between human and microbe. As a result, the immune system may create autoimmunity against them and attack "self" tissue.

*Source: Nicolson, G. Rheumatoid Arthritis, Multiple Sclerosis, Lupus, Inflammatory Bowel Diseases, Scleroderma and other Autoimmune and Degenerative Diseases, http://www.immed.org/illness/autoimmune_illness_research.html; [A M Ercolini](#) and [S D Miller](#). The role of infections in autoimmune disease. *Clin Exp Immunol.* 2009 Jan; 155(1): 1–15; http://www.immed.org/illness/fatigue_illness_research.html*

Laboratory tests for *Mycoplasma pneumoniae*

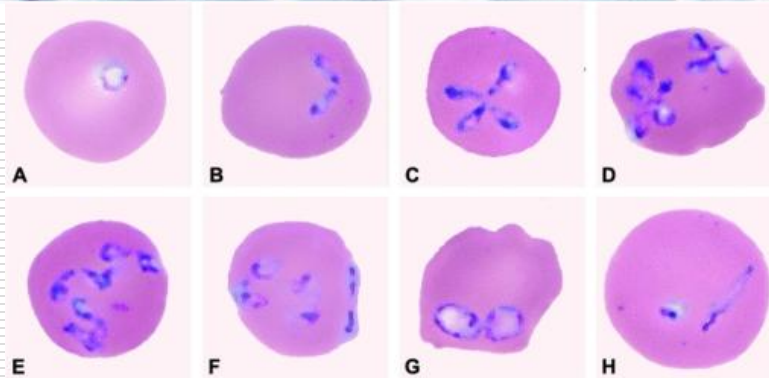
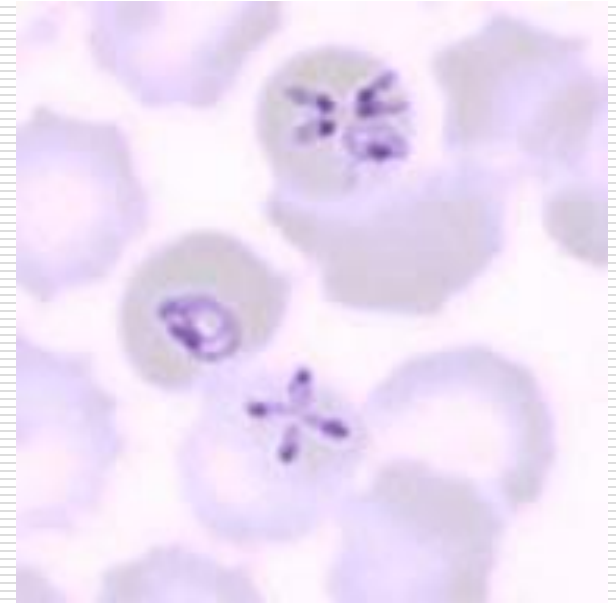
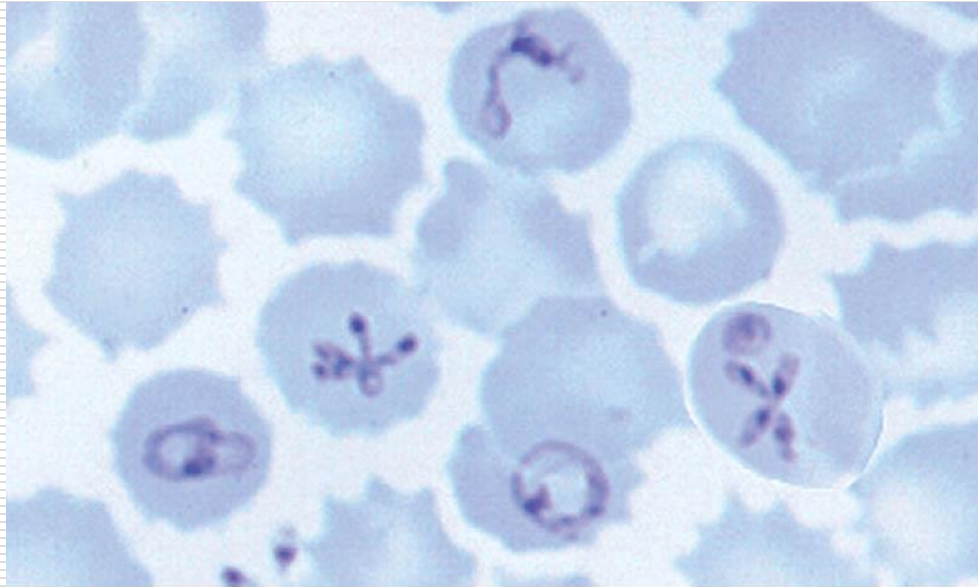
***Mycoplasma pneumoniae* IgA** because of its presence on mucosal membranes (the cilia!)

***Mycoplasma pneumoniae* IgG antibodies**
(half-life of local IgA antibodies: 2 weeks)

***Mycoplasma pneumoniae* EliSpot** **NEW**

Mycoplasma pneumoniae PCR or bacterial culture in blood/sputum/secretion

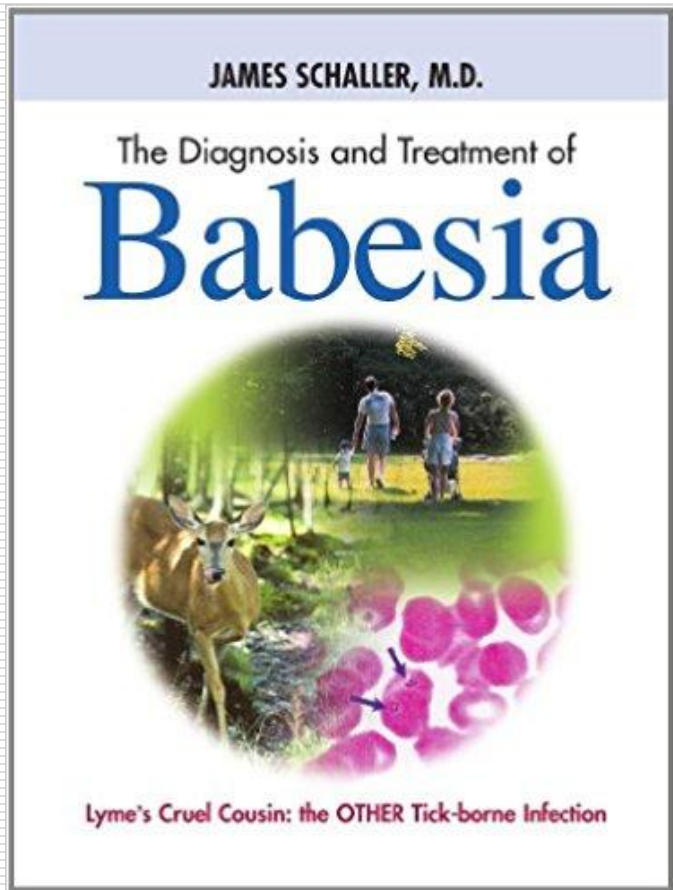
Babesia: a parasite that targets host erythrocytes



**Babesia parasites infect red blood cells, forming a “Maltese Cross”:
babesiosis can cause haemolytic anaemia (from the destruction of red blood cells)**

Source: Lynne Garcia LSG and Associates; <https://www.cdc.gov/dpdx/babesiosis/index.html>; <http://www.sciencedirect.com/science/article/pii/S2213224412000077>

“Lyme’s Cruel Cousin”



Immediately enters the erythrocytes, similar to malaria. Is slow growing and occurs in low concentrations in the body, so is harder to eradicate than malaria. Can be a silent infection – may persist for many years. Most common strains are microti, divergin and duncanci (WA-1).

Forms erythrocyte clusters (= increases clotting) as a source of food, and for protection from the immune system. Enlarged red blood cells, swollen with organisms, can impede passage through capillaries; **can result in spontaneous rupture of small blood vessels, causing bruising/ petechiae (small red spots on the skin); intravascular coagulation can greatly slow/reduce transcutaneous oxygen penetration**

Source: Schaller, James, “The Diagnosis and Treatment of Babesia”, 2006, Hope Academic Press; Vannier E. et al. Human Babesiosis. *Infect Dis Clin North Am.* 2008 Sep; 22(3): 469–ix.

Babesia: Symptoms

Commonly associated with severe Babesia:

- **High fever (105° F)**
- **Severe fatigue and malaise**
- **Shaking chills**
- **Recurrent severe drenching sweats, esp. at night**
- **Severe headache**
- **Muscle aches (myalgia)**
- **Joint pain (arthralgia)**
- **GI: nausea, abdominal pain, diarrhea**
- **Decreased blood pressure**
- **Jaundice (probably due to lysis of red blood cells)**
- **Bruising**
- **Petechiae**
- **Decreased cognition (from intravascular coagulation)**
- **Dark urine**
- **Pulmonary oedema**

Source: Vannier E. et al. Human Babesiosis. Infect Dis Clin North Am. 2008 Sep; 22(3): 469–ix.; Akel T, Mobarakai, N. Hematologic manifestations of babesiosis. Annals of Clinical Microbiology and Antimicrobials 2017;16:6

Babesia: Signs/abnormal labs

Thrombocytopenia (decreased platelets)

- **Enlarged spleen and liver**
- **Anaemia (low haemoglobin)**
- **Evidence of haemolysis (destruction of red blood cells)**
- **Elevated liver function**
- **Mild neutropenia/leukopenia (decreased white blood cells)**
- **Low or unstable blood pressure**
- **Organ malfunction (heart attack, stroke, respiratory distress, kidney failure)**

Source: Rosenblatt JE et al. Laboratory Diagnosis of Infections Due to Blood and Tissue Parasites. Clinical Infectious Diseases, Volume 49, Issue 7, 1 October 2009, Pages 1103–1108

Immune system manipulation

Like other stealth microbes, **Babesia manipulates cytokines** (chemical messengers) of the immune system to its own benefit.

At initial infection, Babesia immediately stimulates IL-10. This has the effect of downregulating all of the Th1 cytokines, reducing l-arginine (Babesia creates a state of l-arginine depletion), therefore inhibiting nitric oxide (NO) production. IFN-gamma also stimulates NO production. **Decreasing NO is one of the major actions of the organism in the body.**

Source: Jeong Y et al. Induction of IL-10-Producing CD1d^{high}CD5⁺ Regulatory B Cells following Babesia microti-Infection. <https://doi.org/10.1371/journal.pone.0046553>; Dede S et al. Oxidation Products of Nitric Oxide and the Concentrations of Antioxidant Vitamins in Parasitized Goats. ACTA VET. BRNO 2002, 71: 341–345; Stich RW et al. Stimulation of nitric oxide production in macrophages by Babesia bovis. [Infect Immun.](#) 1998 Sep;66(9):4130-6.

Laboratory tests for Babesia

Babesia microti IgG/IgM antibodies

Babesia microti EliSpot

NEW

Babesia DNA PCR or FISH in blood (EDTA blood)

Blood smear

Less commonly:

- Haemolytic anaemia (erythrocytes, haptoglobin)
- Thrombocytopenia
- Leucocytopenia
- Elevated liver enzymes (ALT, AST, GGT)
- Elevated creatinine, urea
- Haemoglobinuria

Bartonella: A gram-negative stealth bacterium

JAMES SCHALLER, M.D.

Bartonella: Diagnosis and Treatment

This Book Could Save Your Life!



Heart Attacks | Rage | Fatigue | Agitation | Brain Fog
Migraines | Bipolar Disorder | Rigidity | Addictions
Personality Change | Obesity | Depression
Eye Problems | Unusual Edema | Memory Loss

Lyme Disease's Cruel Cousin

Found in Ticks, Fleas, Pet Saliva, Lice and Dust Mites

FULL COLOR EDITION - PART ONE

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“Bartonella is one of the most serious infections in the world ... a profoundly dangerous stealth infection ... and unknown to 99.9% of the world’s clinical and academic health care workers”

Bartonella: action on endothelial tissue

As soon as the bacteria enter the bloodstream, they immediately colonise 4 sites: the red blood cells, the spleen, the liver, and the bone marrow. From the bone marrow, it enters CD34+ cells generated there which are the progenitors of endothelial and red blood cells.

Bartonella live primarily inside the lining of the blood vessels: endothelial cells, and its prime aim is to scavenge nutrients from blood cells. The CD34+ cells immediately take the Bartonella to locations throughout the body where endothelial inflammation is already occurring. This means of course that it tends to go to areas that are already weak. The bacteria use epidermal growth factor (VEGF) to cause endothelial overgrowth in order to make the largest possible area for themselves. But hyperproliferation of endothelial tissue can lead to narrowing of the arteries, similar to in atherosclerosis – it can even block blood vessels.

Source: Dehio C. Bartonella interactions with endothelial cells and erythrocytes. [Trends Microbiol.](#) 2001 Jun;9(6):279-85. Kempf VA. Evidence of a leading role for VEGF in Bartonella henselae-induced endothelial cell proliferations. [Cell Microbiol.](#) 2001 Sep;3(9):623-32

Bartonella: Etiology of the symptoms

Where the endothelial growth occurs in the body is where symptoms occur. In the eye: ocular bartonellosis. Kidneys: renal bartonellosis. Heart: cardiac bartonellosis. It also uses IL-8 to decrease circulating neutrophils, causing neutropenia. This gives the bacteria time to enter into specific cells and hide from the immune system. The IL-8 also causes a clumping together of infected cells.

What they want specifically from the red blood cells is their heme – they cause oxidisation of the haemoglobin in the red blood cells to methemoglobin. While this feeds the Bartonella the heme and iron they so crave, for the host, this can cause severe symptoms because methemoglobin cannot bind oxygen, unlike haemoglobin. So you can imagine the repercussions: air hunger, headache, fatigue, anxiety, dizziness, palpitations, arrhythmias and seizures.

Source: Buhner, SH. Healing Lyme Disease Coinfections: Complementary and Holistic Treatments for Bartonella and Mycoplasma. Healing Arts Press; McCord AM et al. Autocrine Role for Interleukin-8 in Bartonella henselae-Induced Angiogenesis. [Infect Immun](#). 2006 Sep; 74(9): 5185–5190

Bartonella: Manifestations

Lymph: Frequently the cardinal symptom – enlargement, tenderness, leukocytosis, fever

Ocular: Redness, blurred vision, retinitis, uveitis, optic neuritis, etc.

Ear: Otitis media, vertigo, labyrinthitis

Neurological issues: Headaches, encephalopathy, seizures, facial nerve paralysis, neuralgia, panic attacks, muscle spasms, unexplained rage, multiple other symptoms

Hepatosplenic issues: Hepatosplenomegaly, abscesses/granulomas in liver/spleen, gallbladder may be involved

Renal issues: Glomerulonephritis, microabscesses, urogenital pain, proteinuria, dark urine

Orthopaedic: Bone infection: pain, osteomyelitis, arthritis. Knee, wrist, elbow, ankle, bunion; spine and pelvic girdle. Myalgia, tendonitis.

Skin: Rashes, striae, non-healing ulceration, purpuras, vasculitis

Source: Berghoff W. Chronic Lyme Disease and Co-infections: Differential Diagnosis. [Open Neurol J. 2012; 6: 158–178.](#)

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3565243/table/T3/>; Buhner, SH. Healing Lyme Disease Coinfections: Complementary and Holistic Treatments for Bartonella and Mycoplasma. Healing Arts Press

Bartonella: Striae



Source: Identification of Novel Zoonotic Activity of Bartonella spp., France. Emerging Infectious Disease Journal – CDC. Volume 22, Number 3 –March 2016.

Bartonella: Vectors

Transmitted by any biting insects such as fleas, Ixodes ricinus ticks (Germany/Europe: up to 40% of ticks are contaminated), mosquitoes, sandflies, lice, chiggers, biting flies, scabies, mites, and even louse-eating spiders), but can **also be transmitted by contaminated bites (of animals), scratch (cat scratch), and ingestion.**

Beyond cats, *B. henselae* is also carried by dogs and other mammals including humans.

B. quintana, spread by body lice, *B. quintana* has also been found in cats, dogs, monkeys, gerbils, rats, and can also be transmitting by other insect vectors.

Source: <https://www.cdc.gov/bartonella/transmission/index.html>; Billeter SA et al. Vector transmission of Bartonella species with emphasis on the potential for tick transmission. *Med Vet Entomol.* 2008 Mar;22(1):1-15.

Laboratory tests Bartonella

Bartonella henselae IgG/IgM antibodies

Bartonella quintana IgG/IgM antibodies

Bartonella henselae EliSpot

NEW

Bartonella PCR in blood (EDTA)

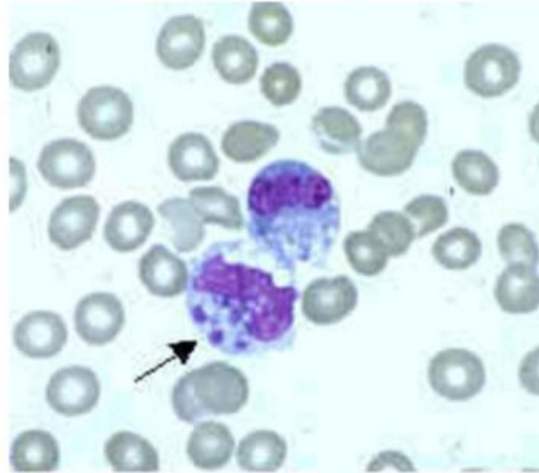
Histology: PCR on biopsies (striae/haemangioma/lymphadenitis)

Elevated vascular endothelial growth factor (VEGF): seldom increased, but if it is, it can be used as an activity marker for monitoring

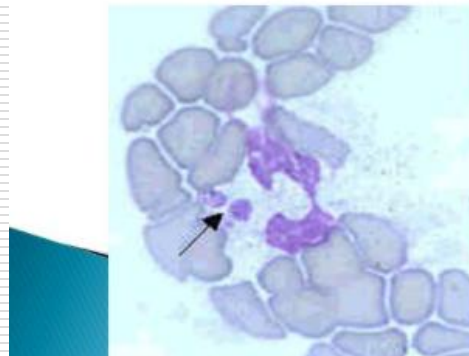
Ehrlichia/Anaplasma: rod shaped bacteria that live inside white blood cells

Small intracellular gram-negative bacteria that cannot exist outside host cells.

Ehrlichia prefers mononuclear phagocytes, while Anaplasma prefers neutrophils



Ehrlichia chaffeensis primarily infects mononuclear leukocytes (predominantly monocytes and macrophages),



The pathogen that causes human granulocytic ehrlichiosis (HGE) (Anaplasmosis) primarily infects granulocytes (neutrophils and rarely eosinophils).

Source: https://en.wikipedia.org/wiki/Ehrlichia_chaffeensis;

Ehrlichia: spread/symptoms

Most commonly infects monocytes and macrophages. The bacteria increase granulocytes in the spleen tenfold – this swells the spleen. The natural killer cells are disproportionately distributed to the liver where they produce inflammation, so the liver enzymes AST are elevated in 90% of cases/ALT in 84%. Cholestasis is quite common, sometimes with bile duct injury.

Vectors: Ticks, possibly fleas (cats/dogs)

Symptoms: (incubation time: days up to 4 weeks): rapid onset of beginning illness with fever, headache and prostration, headaches are "sharp, knife-like and often located behind the eyes", muscle pain, not joint pain, neurological symptoms, psychiatric symptoms, rarely: diffuse vasculitic rash, including palms and soles (<10%)

Anaplasma: spread/symptoms

Most commonly infects neutrophils.

Most commonly affected organs/tissues: bone marrow, erythrocytes, liver, spleen, lymph system, lungs

Men more commonly affected than women (2:1).

Markers: Leukopenia (72%), thrombocytopenia (73%), raised AST/ALT, anaemia (55%)

Symptoms: Similar to Ehrlichia, but central nervous system involvement less common than with HME

Vectors: Ticks (Ixodes, Dermacentor, Rhipicephalus, Haemaphysalis Amblyomma), person to person transmission, through respiratory secretions (& through blood transfusion/bone marrow transfer)

Source: Buhner, SH. Healing Lyme Disease Coinfections: Complementary and Holistic Treatments for Bartonella and Mycoplasma. Healing Arts Press

Diagnosis of Ehrlichia/ Anaplasma

Ehrlichia/Anaplasma Elispot-LTT

Ehrlichia/Anaplasma DNA-PCR in blood (EDTA blood): direct detection

Bacteria detection in Giemsa blood smear

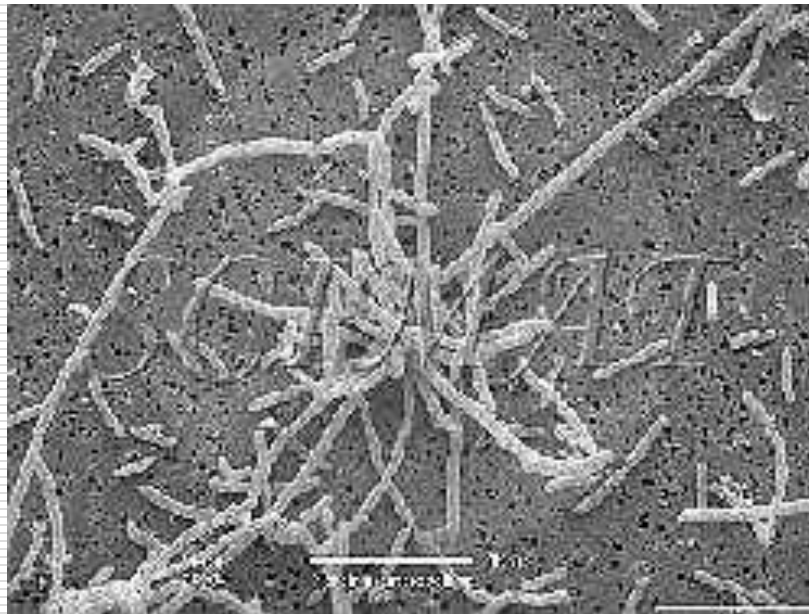
Ehrlichia-IgM and Ehrlichia-IgG antibodies

Leucopenia / Thrombocytopenia / Anaemia

Elevated liver enzymes

Yersinia enteropathica

Yersinia is an enteropathic bacterium. It penetrates the intestinal wall and the mesenteric lymph nodes. Several ectoparasites including ticks have been found to be infected with Yersinia – the most common vectors are rodents and fleas.



Yersinia: Destruction of Peyer's Patches

The follicle-associated epithelium (FAE) is the primary site of host-pathogen interaction. ... At day 7 after infection, the cytoarchitecture of the infected PP was almost completely destroyed and yersinia-induced abscesses often replaced the lymphoid follicles entirely

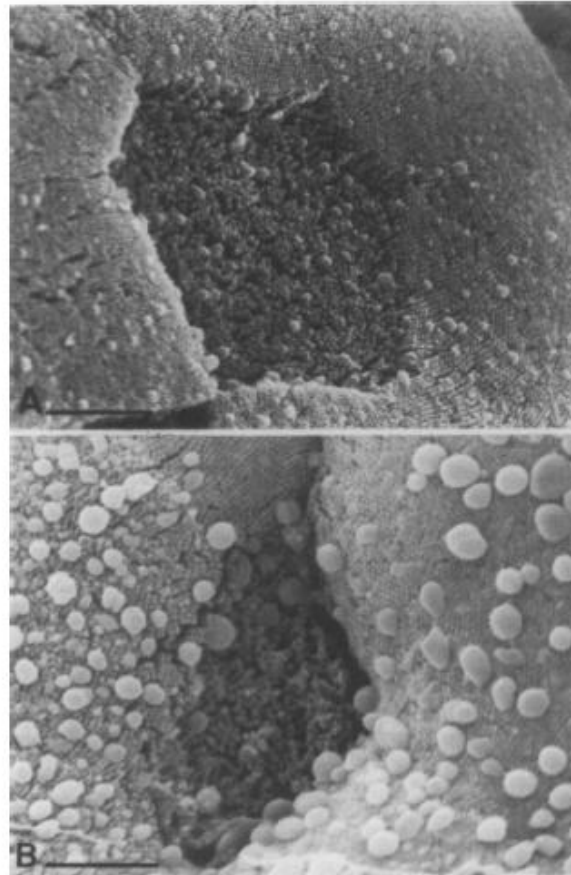


Fig. 2. SEM of M cell within the FAE. A, Normal, uninfected mice, bar = 2 μ m; B, 24 h after orogastric infection with 10^{10} *Y. enterocolitica*, many bubble like structures/vesicles on the surface, bar = 2 μ m.

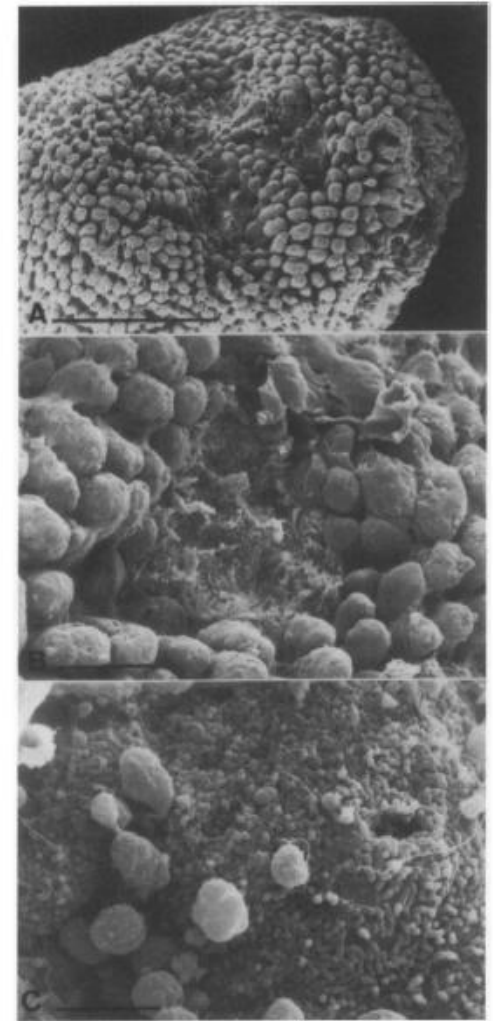


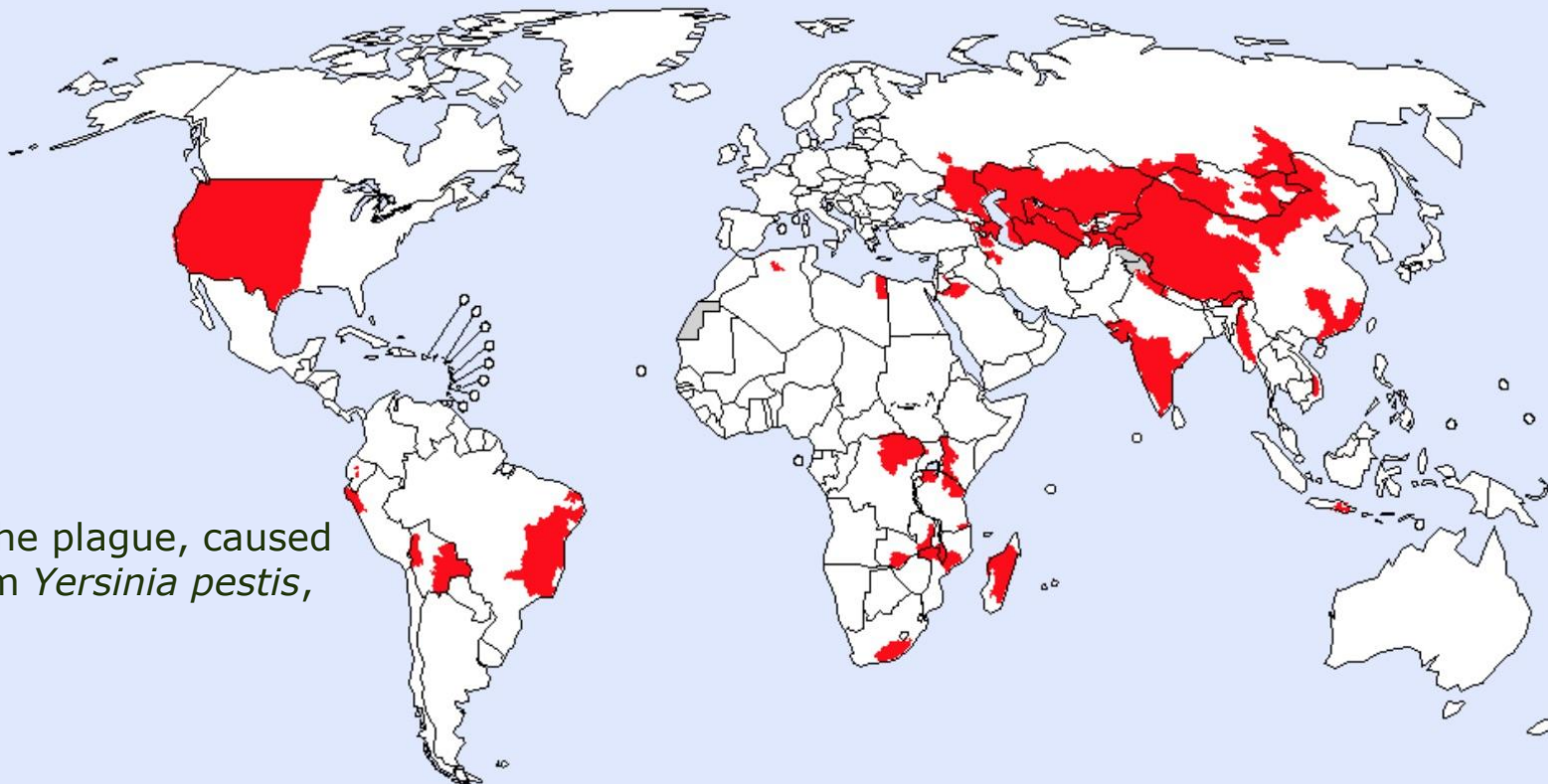
Fig. 3. SEM of the luminal, surface of PP 48 h after orogastric infection with 10^{10} *Y. enterocolitica*. A, PP with domes, bar = 1 mm; B, alteration of the luminal surface of a dome and adjacent villi, bar = 200 μ m; C, FAE with bacteria and remnants (possibly nuclei) of cells, enterocytes and M cells cannot be identified, bar = 10 μ m.

Source: [Autenrieth IB¹](#), [Firsching R](#). Penetration of M cells and destruction of Peyer's patches by *Yersinia enterocolitica*: an ultrastructural and histological study. *J Med Microbiol*. 1996 Apr;44(4):285-94

Yersinia pestis – e.g. Madagascar

Since August 2017, Madagascar is experiencing a large outbreak of plague affecting major cities and other non-endemic areas.

From 1 August through 30 October 2017, a total of 1801 confirmed, probable and suspected cases of plague, including 127 deaths, have been reported by the Ministry of Health of Madagascar to WHO. ...



Distribution of the plague, caused by the bacterium *Yersinia pestis*, in 2016

Symptoms of Yersinia

Table 7. Yersiniosis (*Y. enterocolitica*) Symptomatology

Stages	Symptoms
Early stage	Gradual development of gastroenteritis, pharyngitis, complications due to inflammation of the intestinal wall, mesenteric lymphadenopathy. Excretory for months after abatement of gastroenteritis
Late stage	Articular manifestations: Reactive arthritis in hip, knee, upper ankle, sacroiliac joints, arthralgias, ankylosing spondylitis, rheumatoid arthritis, sacroiliitis Erythema nodosum, iridocyclitis, conjunctivitis, gastrointestinal complaints, abdominal pain, diarrhea, ulcerative colitis, nephritis, insulin-dependent diabetes mellitus, hepatitis (ANA positive, rheumatoid factor positive), myocarditis (rare), central and peripheral nervous system manifestations, multisystem disease Disease progression in stages with intervals of fewer complaints Correlation with thyroiditis Positive LTT Oscillating serological findings (correlation with disease expression)

Source: Berghoff W. Chronic Lyme Disease and Co-infections: Differential Diagnosis. *Open Neurol J.* 2012; 6: 158–178

Diagnosis of Yersinia

Yersinia Elispot-LTT

Ehrlichia/Anaplasma-DNA-PCR in blood (EDTA blood): direct detection

Yersinia IgM and IgG antibodies

The Elispot

The benefits of enzyme-linked Immunospot (Elispot) testing

Source: <http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/pulmonary/sarcoidosis/>

Evidence-based literature on false seronegativity

- Klempner MS, Schmid CH, Hu L, Steere AC, Johnson G, McCloud B, Noring R, Weinstein A. Intralaboratory reliability of serologic and urine testing for Lyme disease. *Am J Med.* 2001 Feb 15;110(3):217-9.
- Banyas GT. Difficulties with Lyme serology. *J Am Optom Assoc.* 1992 Feb;63(2):135-9.
- Faller J, Thompson F, Hamilton W. Foot and ankle disorders resulting from Lyme disease. *Foot Ankle.* 1991 Feb;11(4):236-8.
- Nields JA, Kueton JF. Tullio phenomenon and seronegative Lyme borreliosis. *Lancet.* 1991 Jul 13;338(8759):128-9.
- Schutzer SE, Coyle PK, Belman AL, Golightly MG, Drulle J. Sequestration of antibody to *Borrelia burgdorferi* in immune complexes in seronegative Lyme disease. *Lancet.* 1990 Feb 10;335(8685):312-5.
- Paul A. [Arthritis, headache, facial paralysis. Despite negative laboratory tests *Borrelia* can still be the cause]. *MMW Fortschr. Med* 2001 Feb 8;143(6):17.
- Ang CW, Notermans DW, Hommes M, Simoons-Smit AM, Herremans T. Large differences between test strategies for the detection of anti-*Borrelia* antibodies are revealed by comparing eight ELISAs and five immunoblots. *Eur J Clin Microbiol Infect Dis.* Published online: 27 Jan 2011
- Wojciechowska-Koszko et al.: Serodiagnosis of borreliosis: indirect immunofluorescence assay, enzyme-linked immunosorbent assay and immunoblotting. *Arch Immunol Ther Exp (Warsz.)* 2011 Feb;59(1):69-77. Epub 2011 Jan 22.
- Durovska J. et al.: Our experience with examination of antibodies against antigens of *Borrelia burgdorferi* in patients with suspected lyme disease. *Bratisl Lek Listy.* 2010;111(3):153-5.

False seronegativity is a significant issue with antibody testing (humoral/adaptive immune system)

Seronegativity in Lyme borreliosis and Other Spirochetal Infections

103 articles in this collection alone

“If false results are to be feared, it is the false negative result which holds the greatest peril for the patient.”

Gestational Lyme borreliosis. Implications for the fetus. MacDonald AB. *Rheum Dis Clin North Am*, 15(4):657-77. 1989.

<i>Author</i>	<i>Year</i>	<i>Title</i>	<i>Journal</i>
<i>Borrelia burgdorferi</i>			
1. Dejmkova H; Hulinska D; Tegzova D; Pavelka K; Gatterova J; Vavrik P.	2002	Seronegative Lyme arthritis caused by <i>Borrelia garinii</i>. <i>[From the abstract:] "A case of a female patient suffering from Lyme arthritis (LA) without elevated antibody levels to <i>Borrelia burgdorferi sensu lato</i> is reported. Seronegative Lyme arthritis was diagnosed based on the classic clinical manifestations and DNA-detected <i>Borrelia garinii</i> in blood and synovial fluid of the patient, after all other possible causes of the disease had been ruled out. The disease was resistant to the first treatment with antibacterial agents. Six months after the therapy, arthritis still persisted and DNA of <i>Borrelia garinii</i> was repeatedly detected in the synovial fluid and the tissue of the patient. At the same time, antigens or parts of spirochaetes were detected by electron microscopy in the synovial fluid, the tissue and the blood of the patient. The patient was then repeatedly treated by antibiotics and synovectomy has been performed."</i>	Clinical Rheumatology, 21(4):330-4
2. Tylewska-Wierzbawska S; Chmielewski T;	2002	Limitation of serologic testing for Lyme borreliosis: evaluation of ELISA and western blot in comparison with PCR and culture methods. <i>[From the abstract:] "No correlation was found between levels of specific <i>B. burgdorferi</i> antibodies detected with a recombinant antigen ELISA and the number of protein fractions developed with these antibodies by immunoblot. Moreover, Lyme borreliosis patients who have live spirochetes in body fluids have low or negative levels of borrelial antibodies in their sera. This indicates that an efficient diagnosis of Lyme borreliosis has to be based on a combination of various techniques such as serology, PCR and culture, not solely on serology." [Testing was performed on samples from 90 patients.]</i>	Wien Klin Wochenschr, 114(13-14):601-5
3. Breier F; Khanakah G; Stanek G; Kunz G; Aberer E; Schmidt B; Tappeiner G.	2001	Isolation and polymerase chain reaction typing of <i>Borrelia afzelii</i> from a skin lesion in a seronegative patient with generalized ulcerating bullous lichen sclerosus et atrophicus. <i>[From the abstract:] "Spirochaetes were isolated from skin cultures obtained from enlarging LSA lesions. These spirochaetes were identified as <i>Borrelia afzelii</i> by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and polymerase chain reaction (PCR) analyses. However, serology for <i>B. burgdorferi sensu lato</i> was repeatedly negative."</i>	Br J Dermatol, 144(2):387-392
4. Brunner M.	2001	New method for detection of <i>Borrelia burgdorferi</i> antigen complexed to antibody in seronegative Lyme disease. <i>[From the abstract:] "...serologic tests for early Lyme disease can be falsely negative due to lack of sensitivity of ELISAs and Western blots. Most routine antibody tests are designed to detect free antibodies, and in early, active disease, circulating antibodies may not be free in serum but sequestered in complexes with the antigens which originally triggered their production. This difficulty may be overcome by first isolating immune complexes (IC) from the serum and using this fraction for testing. Free <i>Borrelia</i>-specific antibodies can then be liberated from the immune complexes which may enhance test sensitivity in patients with active disease. We developed a technique that captures the antibody component of IC on immunobeads, and subsequently releases the antigen component of IC. Immunoblotting with monoclonal antibody detected at least one antigen to be OspA, thus definitively demonstrating a <i>Borrelia</i>-specific antigen in circulating IC in early Lyme disease. This test is also useful in demonstrating Bb antigen in otherwise seronegative Lyme disease patients."</i>	J Immunol Methods, 249(1-2):185-190

<i>Author</i>	<i>Year</i>	<i>Title</i>	<i>Journal</i>
39. Pachner A.	1995	Early disseminated Lyme disease.	Am J Med, 98 (suppl 4A):4A-30S-51S – Discussion.
<p><i>"The correlation between a positive Western blot and Lyme arthritis is probably the best of almost any Western blot and any Lyme disease manifestation. With neurologic disease, I have had a lot of patients who don't have a positive Western blot; they just have not developed a peripheral antibody response, for whatever reason."</i></p>			
40. Coyle PK; Schutzer SE; Deng Z; Krupp LB; Belman MD; Benach JL; Luft BJ.	1995	Detection of <i>Borrelia burgdorferi</i> -specific antigen in antibody negative cerebrospinal fluid in neurologic Lyme disease.	Neurology, 45:2010-2014
<p><i>[From the abstract:] "RESULTS: Of the 35 of 83 (42%) patients who were positive for OspA antigen in their CSF, 15 (43%) were antigen positive despite being antibody-negative in CSF. Seven of these 15 (47%) had otherwise normal routine CSF analyses. Six of these 15 (40%) patients met strict CDC surveillance criteria for Lyme disease: four (27%) patients had seroconversion coincident with new neurologic problems; and three (20%) with characteristic syndromes for Lyme disease were seronegative, but had complexed antibody to B. burgdorferi. The final two patients (13%) were seropositive and had unexplained neurologic problems not characteristic of Lyme disease. CONCLUSIONS: B. burgdorferi antigen can be detected in CSF that is otherwise normal by conventional methodology, and can be present without positive CSF antibody. Since CSF antigen implies intrathecal seeding of the infection, the diagnosis of neurologic infection by B. burgdorferi should not be excluded solely on the basis of normal routine CSF or negative CSF antibody analyses."</i></p> <p><i>[From the article:] "Prompt and precise diagnosis is difficult because basic microbiologic tests such as culture and staining have not been useful, on a broad scale, to document the presence of the spirochete in a body fluid. Instead, detection of specific antibodies to B burgdorferi in blood and CSF is commonly used to support or refute a clinical suspicion of infection. Many of the commercially available assays have been plagued by lack of sensitivity, specificity, and reproducibility. Furthermore, the absence of free antibodies to B burgdorferi components has been documented in well-characterized erythema-migrans-positive cases of Lyme disease, including those with prominent neurologic involvement."</i></p>			
41. Karma A; Seppala I; Mikkila H; Kaakkola S; Viljanen M; Tarkkanen A.	1995	Diagnosis and clinical characteristics of ocular Lyme borreliosis.	American Journal of Ophthalmology, 119(2):127-35
<p><i>[From the abstract:] "Results of ELISA disclosed that five patients [out of ten] were seropositive, two patients showed borderline reactivity, and three patients were seronegative. Four of the five patients with borderline or negative results by ELISA had a positive result by western blot analysis. ... CONCLUSIONS: Late-phase ocular Lyme borreliosis is probably underdiagnosed because of weak seropositivity or seronegativity in ELISA assays."</i></p>			
42. Lawrence C; Lipton RB; Lowy FD; Coyle PK.	1995	Seronegative chronic relapsing neuroborreliosis.	European Neurology, 35(2):113-7
<p><i>[From the abstract:] This article reports a Lyme disease patient "who experienced repeated neurologic relapses despite aggressive antibiotic therapy." The patient was seronegative. "Although the patient never had detectable free antibodies to B. burgdorferi in serum or spinal fluid, the CSF was positive on multiple occasions for complexed anti-B. burgdorferi antibodies, B. burgdorferi nucleic acids and free antigen."</i></p>			
43. Millner M.	1995	Neurologic manifestations of Lyme borreliosis in children.	Wiener Medizinische Wochenschrift, 145(7-8):178-82
<p><i>"Our own observations in children which suffered from an acute neuroborreliosis (NB) showed the following:... Indeed, there is a seronegative NB also in children."</i></p>			

The Elispot technique

Using T-cells to show a cellular response against antigens is very sensitive, and indicates active infection (in contrast to antibodies, which can remain for months or years, long after an infection is gone).

EliSpot (enzyme-linked immunosorbent spot) technology has long been used in Germany to do exactly this: it quantifies T-cells that secrete signature proteins (such as a given cytokine) against a specific antigen.

“The quantification of single cell interferon-gamma (IFN- γ) release for assessing cellular immune responses using the Enzyme-linked immunospot (ELISPOT) assay is an invaluable technique in immunology.”¹

Source: 1 [Sedegah M.](#) The Ex Vivo IFN- γ Enzyme-Linked Immunospot (ELISpot) Assay [Methods Mol Biol.](#) 2015;1325:197-205

ELISPOT: New T-Cell Test a Game Changer for Lyme Disease

- ... The sensitivity of the ELISPOT is estimated at 84%, and the specificity is 94%...
- ... ELISPOT assays provide robust, highly reproducible data...
- ... ELISPOT can be retested to gain additional information in follow-up assays...
- ... the tests in the two-assay system (ELISPOT + CD57 cell count) complement each other in the quest to understand T cell-mediated immunity in vivo....

Source: Lehman PV et al.: Unique Strengths of ELISPOT for T Cell Diagnostics in: Kalyuzhny AE. Handbook of ELISPOT:Methods and Protocols, Methods in Molecular Biology, Vol. 792. 2nd Ed: Springer; 2012: 3-23

94 % **Specificity of Borrelia Elispot-LTT**

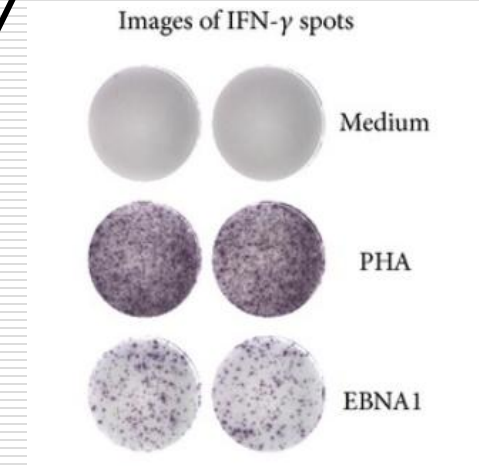
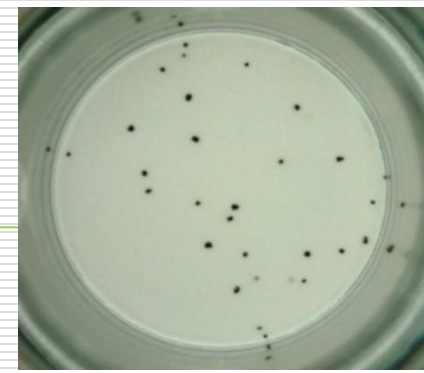
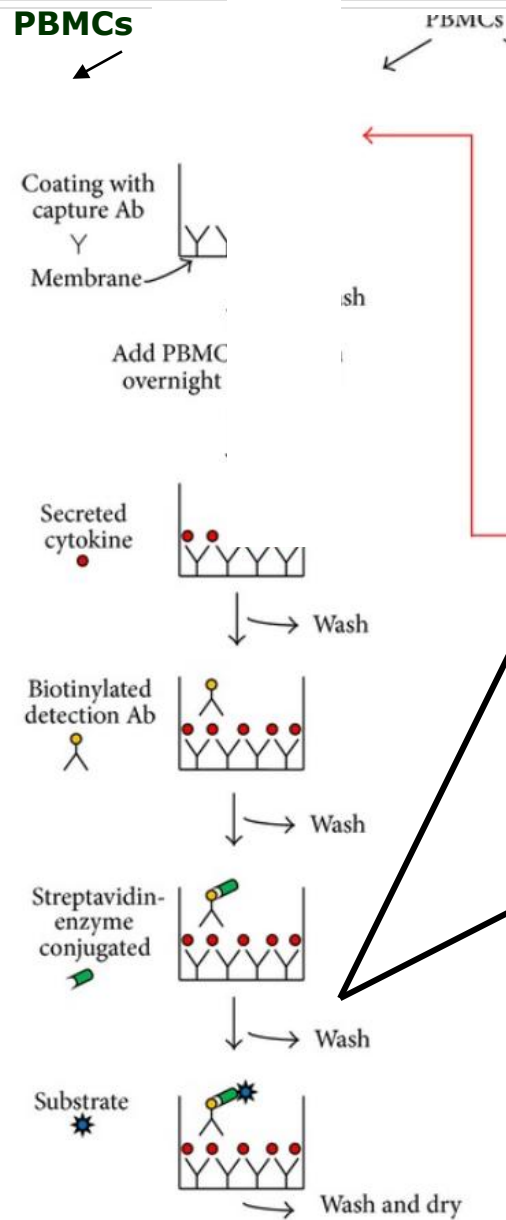
84 % **Sensitivity of Borrelia Elispot-LTT**



ELISpot assay

Fig. 1. The standard ELISPOT is used to measure antigen-specific effector T cells. The membrane's plate is coated with an antibody (Ab) specific for the cytokine of interest (capture Ab). Peripheral blood mononuclear cells (PBMCs) are added and incubated overnight. The cytokine released by the cells binds to the capture Ab. After washing, an anti-cytokine biotinylated detection Ab is added followed by streptavidin conjugated with an enzyme. Finally, an enzyme substrate is added to produce coloured spots.

Source: [Calarota SA](#). Enumeration and characterization of human memory T cells by enzyme-linked immunospot assays. *Clin Dev Immunol*. 2013;2013:637649



Spots are counted using an automated ELISPOT reader

Technique: further details

“The enzyme-linked immunosorbent spot (ELISpot) assay is commonly used for the identification and enumeration of cytokine-producing cells with exquisite sensitivity at the single-cell level.

ELISpot is a highly sensitive method in immunology to enumerate cells producing a given cytokine. Cells are stimulated in a microtiter plate pre-coated with a specific anti-analyte antibody. In response to the stimulation, T cells specifically release IFN γ , that are bound to the anti-analyte antibody. After a washing step, which removes the cells from the wells, the location of the cytokine-releasing cell is visualized by an enzyme-labeled detection antibody and its corresponding chromogenic substrate. The end result is a set of coloured spots, each of which represents an area where a cell secreting IFN γ had been located.”

Source: Navarrete MA ELISpot and DC-ELISpot Assay to Measure Frequency of Antigen-Specific IFN γ -Secreting Cells, in Hnasko R (Editor), Elisa Methods and Protocols 2015. https://link.springer.com/protocol/10.1007/978-1-4939-2742-5_8

References on the Elispot: examples

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Navarrete MA, Bertinetti-Lapatki C, Michelfelder I et al (2013) Usage of standardized antigen-presenting cells improves ELISpot performance for complex protein antigens. J Immunol Methods 391:146–153

Czerkinsky CC, Nilsson LA, Nygren H et al (1983) A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J Immunol Methods 65:109–121

Keilholz U, Weber J, Finke JH et al (2002) Immunologic monitoring of cancer vaccine therapy: results of a workshop sponsored by the Society for Biological Therapy. J Immunother 25:97–138
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Calarota SA. Enumeration and characterization of human memory T cells by enzyme-linked immunospot assays. *Clin Dev Immunol.* 2013;2013:637649

Currently the EliSpot is available for:

- Borrelia burgdorferi (B.b. sensu stricto + garinii + afzelii)
- Borrelia myamotoi
- Ehrlichia/Anaplasma
- Bartonella henselae EliSpot** **NEW**
- Babesia microti EliSpot** **NEW**
- Chlamydia pneumoniae
- Chlamydia trachomatis
- Mycoplasma pneumoniae** **NEW**
- Yersinia species
- Epstein Barr Virus (EBV)
- Cytomegalovirus (CMV)
- Herpes Simplex Virus 1 / 2 (HSV 1 / 2)
- Varicella Zoster Virus (VZV)** **NEW**

Thank you very much for your attention !

Armin Schwarzbach Ph.D.

**Medical Doctor and
Specialist for laboratory medicine**

