

Research Article

A simple method for the detection of live *Borrelia* spirochaetes in human blood using classical microscopy techniques

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ABSTRACT

We have developed a simple method for the detection of live spirochaete stages in blood of patients where chronic borreliosis is suspected. Classic techniques involving phase-contrast and fluorescence microscopy are used. The method is also quite sensitive for detecting other bacteria, protists, fungi and other organisms present in blood samples. It is also useful for monitoring the effects of various antibiotics during treatment. We also present a simple hypothesis for explaining the confusion generated through the interpretation of possible stages of *Borrelia* seen in human blood. We hypothesize that these various stages in the blood stream are derived from secondarily infected tissues and biofilms in the body with low oxygen concentrations. Motile stages transform rapidly into cysts or sometimes penetrate other blood cells including red blood cells (RBCs). The latter are ideal hiding places for less motile stages that take advantage of the host's RBCs blebbing-system. Less motile, morphologically different stages may be passively ejected in the blood plasma from the blebbing RBCs, more or less coated with the host's membrane proteins which prevent detection by immunological methods.

Keywords: spirochaetes; Lyme disease; *Borrelia*; borreliosis; microscopical detection method; human blood

Introduction

Since the discovery of *Borrelia burgdorferi*, the Lyme disease spirochaete, borreliology has evolved from a microbiological curiosity to a major branch of bacterial pathogenesis research. Lyme disease is a global health problem; indeed few infectious diseases have garnered more sustained attention from the scientific and, notably, the lay media [1]. *Borrelia*-related diseases are a major challenge in medical research in many countries of the world, and we refer to comprehensive sources for the history and characteristics of this dangerous disease [2]. The disease is of great relevance today, and will be even more important tomorrow [3].

The most serious challenges are connected to diagnosis. The relatively large *Borrelia*, i.e. *B. burgdorferi*, is in general not readily detectable in blood smears of varying thickness from Lyme disease patients and suspected host animals. Yet blood engorgement from infected host result in up to 100% infected ticks [4].

Spirochaetes in general have been studied among biologists for more than 300 years. Around 1900 it was well documented that some species had a rather complex life-history, including infective resting stages (“cysts”) with very different morphologies deviating from the spiral-shaped form usually depicted from *Spirochaeta gallinarum* [5]. Hindle (op. cit.) has shown from the life-cycle of *S. gallinarum* that the bacteria develop from coccoid bodies inside cells, which they might leave and change to spiral forms that again form coccoid bodies in the blood. Spiral forms may also penetrate blood cells and form such coccoid bodies inside them.

Lyme disease, caused by the spirochaete *B. burgdorferi* presents unusual problems regarding both diagnosis and treatment. A tentative diagnosis from microscopic preparation from many suspected Lyme-diseased human patients has been considered to be quite difficult. Also both detection using DNA techniques and immunological methods often gives false negative results, and/or misdiagnosis. It is further known that the spirochaetes often resist aggressive antibiotic therapy and reappear causing chronic disease, sometimes for many years [6]. The bacterium in blood smears is often only faintly stained with Giemsa-techniques which can lead to false negative results, even in samples where it is known to be present. As a result, substantial evidence has accumulated that numerous patients suffering from chronic borreliosis may not get an adequate diagnosis. Sometimes severely ill patients with suspected borreliosis get neither prompt nor appropriate medical treatment because of the difficulties associated with diagnosis. Here we present a reliable and very simple procedure for detection of live spirochaetes and cysts in the blood by the use of classic techniques in microscopy. The procedure might be carried out in almost any laboratory, provided that necessary precautions against infections of staff are exerted.

Methods and Materials

Instruments

Among necessary instruments we have used a quality phase-contrast microscope, correctly aligned and maintained. (Example a Zeiss Universal microscope with 100 W halogene lamp, phase-contrast condensor 0.90/1.40, apochromatic phase optics, especially a Zeiss Ph 63/1.40 oil immersion lens. Digital cam: for example a Logitech 5000pro or Bresser ocular camera connected to a PC). Methods and instruments are detailed described in [7], [8] and [9].

Chemicals and glassware

Among chemicals and glassware needed are bacteria-free, distilled water, sodium-citrate (i.e. tri-natriumcitrat-2-hydrat ($C_6H_5Na_3O_7 \cdot 2H_2O$) powder, clean microscope slides, standard cover-glasses 24 x 36 mm (eventually 24 x 55 mm) and clean glass Petri-dishes, ca. 20 cm across for incubation of slides.

Laboratory facilities and security measures

As a working environment a basement laboratory with no vibrations is important. A cellar room without windows is to be preferred. No laboratory staff working with other projects should be admitted entrance to the test room. Blood samples from patients with suspected borreliosis are potentially quite dangerous, and strict security measure should be taken. Ticks often transfer other bacteria, protists and dangerous viruses that are very infectious. Staff must always work with protective gloves, and be extremely careful not to cut themselves on sharp objects (e.g. scalpels, slides, etc.), and infectious garbage must be disposed of properly.

Rationale and Results of the Procedure

Extensive experimentation both by various staining techniques and live blood microscopy resulted in a method which we consider reliable for detecting various morphologies of *Borrelia*. It is based upon the following principles:

1. The technique aims to get spirochaetes in the

RBCs (red blood cells) to appear visible after 24-48 hours storage at room temperature.

2. A combination of simulated anoxic conditions and physical forces are used. Slight evaporation of water in the moisture chamber, results in a flattened preparation with expanded (swelled) erythrocytes, sometimes also bacteria. Peripheral blood (fingertip or ear flip) from patients with suspected borreliosis (and close family) should be examined.

3. The microscope preparations form a sort of natural "culture chamber." The idea is that oxygen tension (lower oxygen) should decrease between the slide and cover-glass, which is favorable to *Borrelia* spp replication.

4. Sodium citrate is added, which for unknown reasons seems to further stimulate replication of *Borrelia*.

5. As blood plasma concentrations become low from dilution of sodium citrate solution, excellent optical conditions will be present for examining *Borrelia* in detail within RBCs (Fig. 1). Further blood cells and bacteria become compressed and thin due to surface tension forces by slow evaporation, which enables easy detection.

6. Areas with variations in visibility will appear under the cover-glass throughout a single preparation.

7. *Borrelia* bodies might appear both inside and outside RBCs (Fig. 2 a-f, Fig 4 a-c). White blood cells contain too many structural elements to allow the bacteria to be easily visible.

8. *Borrelia* seems to be sequestered in erythrocytes through various stages of its life cycle, and any structure observed should be closely studied (Fig. 4 a).

9. An acridine-orange solution added to the

preparation show alternating areas with green fluorescence (nucleoid) DNA and orange-red fluorescence cell plasma (RNA-proteins) in filamentous *Borrelia* stages (Fig. 6 a-b).

10. Observations show that the *Borrelia helix* is fragmented in several smaller, sometimes "pearl-like" parts. During a division cycle several, not only two, but several infective "particles" are formed (Fig. 3 b, Fig. 6 a-c).

The technique presented is the result based upon general technical experience for decades [8], [9] and [10], with live cell microscopy of a wide specter of microorganisms, including a four year study of spirochaetes in symbiotic relationships with parabasalid flagellates [11]. They are often difficult to detect by typical microscopy methods.

Procedure

Our results were obtained by the following detailed procedure:

Preparations

A. Clean the fingertip where the blood will be drawn using soap and water, then "sterilize" with a cotton swap moistened with 70% ethanol. Prick with a sterile blood lancet. Collect approx. 20 μ L of blood for each microscope slide with an automatic pipette. The method will also work using blood with EDTA added, for instance blood collected at a health station, provided it is not too old (>1-2 days). It is rare to see contamination from skin bacteria and nevertheless they will *not* be spirochaetes.

B. Place 4-5 clean microscope slides on a clean stainless steel plate.

C. Place a blood droplet approx 6-7 μ l at the center of the lower 1/3 of each microscope slide.

D. Add with another automatic pipette 250 μ l of the sodium citrate solution (0.65g/100 ml dist.

water) at the edge of the blood droplet, touching the droplet.

E. Place on each slide a clean cover-slip (24 x 36 mm) carefully so the blood-sodium-citrate mixture seeps in from the short edge (!) between the cover-glass and the slide. *Do NOT apply any force on the cover-glass of the preparation.*

F. Place slides in a moisture chamber (clean glass Petri-dish with glass lid). In order to avoid excessive drying out of the preparation, place a tiny open glass vial with some water among the slides. *Do NOT cover the bottom with moistened filter paper!*

Leave the prepared microscope slides in the chamber for 24-48 hours. Oxygen levels inside the preparations will decrease creating favorable conditions for spirochaete structures to appear.

G. Areas of expanded erythrocytes will be found, often showing amazing details of inclusions inside, structures not visible by common procedures for blood microscopy. Maximum effects on blood sample are seen after 48 hours. Also stages in the blood plasma appear crisp with high detail.

H. During this procedure the whole preparation becomes extremely flat, and cell components are substantially enhanced. Very crisp and detailed images will appear, clearer than other methods we are aware of.

I. Mix 1:10 000 (w/w) acridine-orange to the sodium citrate solution. Add 1-2 drops to the edge of a 48 hour old preparation. Wait 30 minutes. Bacteria within a certain distance from the cover-glass edge are correctly stained, and show the DNA containing nucleoids as green fluorescing sites evenly scattered among RNA-containing cytoplasm orange-red in a common fluorescence microscope (450 nm excitation, barrier filter 570 nm).

K. By experimenting with the strength of the sodium-citrate solution erythrocytes and various *Borrelia* life-cycle stages can be further expanded, thus showing details that else are only visible in an electron microscope (EM) (Fig. 5 b-f).

Extensive research and experimentation of these techniques have resulted in this extremely simple procedure. It is not only able to detect spirochaetes in an RBC sample, but also to identify the various infective stages occurring during the life-cycle of these species.

Discussion

Varied life cycle stages in human blood

The assumed less motile CWD (cell wall deficient) and other forms [12], [13], [14], have been studied by time-lapse microscopy (for technical see [7], general procedures are described in [8]), which strongly indicate, that these are living structures with some rotational motility. Further “pearl-like” structures, considered to be types of cysts have been observed to germinate (Fig. 5 b), in a process fully in accordance with earlier studies [13]. Our long-term observations thus support the view that all these morphological forms are indeed life cycle stages of *Borrelia*.

Is microscopy of any use in diagnosis?

A substantial number of medical microbiologists and pathologists worldwide, still hold the opinion that diagnosis through microscopy of suspected *Borrelia* infections and chronic borreliosis is unreliable, or of no use at all [6]. Among the arguments presented are that non-helix CWD-forms, small spheres, cysts, “pearl-chain”-like structures, “round-bodies” and filament-like structures are either artifacts from microscopy techniques, protein leakage from blood-cells, fibrine-fibers, or other general changes due to decomposition. We have earlier presented extensive discussions of these problems [13], [14].

It may be argued that the spirochaete-like structures we find are not bacteria based on the findings of numerous biophysical experiments involving erythrocyte blebbing [15]. On the other hand, this phenomenon, which is modeled mathematically, occurs under certain conditions in further aging RBCs. “Pearl-like” or elongated stages may be formed among the RBC blebbing products, and such changes may well be irreversible. The process is hypothesized to represent the result of self-adaption of the membrane surface area to the elastic stress imposed by the spectrin cytoskeleton.

Several morphological and cystic forms

We have thus re-examined the relevant literature including papers written more than 100 years ago, presenting possible life-cycles for spirochaetes including *Borrelia* or close relatives. We fear that if this information is not considered and included in the discussion of the borreliosis problems, little progress in this field will be expected. CWD-forms in *B. burgdorferi* were extensively described in 1993 and later by Margulis and co-workers [16], [17], [18], see also [19]. All these cysts and round bodies presented are live structures. They are assumed to be more or less dormant stages that are able to form active, motile spirochaetes during favorable conditions. It has been proven that the non-motile (or less-motile) stages re-appear from cysts and round bodies which form active, motile spirochaetes during favorable conditions [20]. Change from motile forms to CDW forms have been demonstrated also by [21].

Borrelia in the human blood stream

As seen from Figs. 1-6 these images of *Borrelia* spirochaetes in human blood *do not* resemble cultured specimens on BSK-medium that usually show several helices of small amplitude along the bacterial filament. They are also much less motile. Samples from an extensive material of symbiotic spirochaetes [11] (some thousands slides have been preserved) from termite guts reveal spirochaete shapes nearly

identical to what we see from human blood (example in Fig. 5a). A termite contains in general several different spirochaete species and general observations are most valuable for understanding general morphological diversity which is surprisingly extensive. We have here detected by phase-contrast and fluorescence microscopy *Borrelia* spirochaetes showing formation of several unique and infective CWD-forms (see Fig. 2 a-f).

The Borrelia puzzle

These seemingly diverse facts and controversial observations in the *Borrelia* puzzle may well be explained by the following hypothesis that should be extensively tested by further research:

After the tick bite (or some kind of similar infection locally), motile forms appear and may in some cases migrate to sites with low oxygen tension or other places with favorable growth conditions, (connective tissue in joints, etc., may also sequester the bacteria as biofilms). Those leaking into the blood stream [22], have a risky life. Our own experiments [13] show almost all of them to change into cystic forms within an hour. The helix motile stages are rapidly able to penetrate almost any cell. Some motile spirochaetes thus manage to drill into RBCs. Here they rapidly transform into resting stages protected by these nucleus-deficient cells that provide transport to almost all locations in the body. They escape passively by the RBCs blebbing process in sites with low oxygen concentrations where this process is more likely to occur, and are able, under favourable conditions, to start new infection sites.

Structures suggesting cell wall deficient forms of bacteria were detected in circulating erythrocytes by fluorochrome staining by Pohlod et al. [23]. The second author of this article, Lida Holmes Mattman, presented later evidence that *Borrelia* spirochaetes even prefer RBCs in which they are sequestered. Persistent infections can thus be attributed to the fact that

antibiotics do not easily penetrate the RBCs to target spirochaetes [24].

Here we present images showing less motile *Borellia* inside RBCs, and these hiding sites may play a substantial role in understanding the disease. There is some experimental support for this view [24], [25], [26]. Using microscopy these forms are difficult to study, although they can sometimes be seen by common staining methods. If present they are often overlooked by standard screening methods. In susceptible persons infected RBCs function as additional transport vesicles to other parts of the body, largely protecting the bacterium from antibiotic treatments and the host immune system. Non-motile stages in the blood plasma are largely coated with RBC membrane proteins and may escape detection by immunological-based techniques. Their visibility inside RBCs is hampered mainly by optical problems (negative lens shape of RBCs, refraction index similarity) and staining properties, besides the neglect by observers of unknown shapes and structures of *Borrelia*. Our method causes a kind of inflation of cellular structures, similar to the hypotonic treatment for studying human chromosomes substantially clear up these problems. Motile spirochaetes, on the other hand, leaking out in the blood stream may be attacked by drugs or WBCs, thus slight patient improvements are usual following antibiotic treatment.

Lifelong chronic infection?

The present technique is based upon studies of how live blood samples develop in environments of low oxygen. As mentioned we assume that CWD-forms of this species are likely to persist in tissues of joints, in the nervous system or biofilms lending protection from antibiotics and host immune response. We have further evidence that viable, but less motile cystic stages may hide inside erythrocytes which may well act as carriers to more favorable tissues. If the immune system is stressed due to other causes, infective or not, cysts may come

out of dormancy and the bacterium drills into tissues causing symptoms, an almost perfect evolutionary adaption for surviving long periods in the host [25], [26]. Many people infected with *Borrelia* probably never develop any symptoms of the disease, although they still carry the bacterium in their body. No doubt the bacterium and its hosts have a very long co-evolutionary history. Long term association of symbiotic bacteria in animal tissues tends towards massive gene loss when compared to related bacteria that live freely in the environment. The fact is that the species *Treponema pallidum* and *B. burgdorferi* are no longer free-living but obligate to a host. This implies that in the case of *B. burgdorferi* it might be assumed to be on the road to symbiosis [27].

In the case of *Borrelia*, where the infectious agent resembles a symbiont, complex evolutionary interactions may well exist. In the case of malaria adaptations to an intracellular life style in special target cells are determined by multiple parasite-host interactions. During the periodic waves of fevers and chills the parasite destroys and reinfects RBCs [28]. Even if little or nothing is known about the advantages for hosts having chronic *Borrelia* infections without symptoms, we suspect that the intracellular *Borrelia* stages in RBCs may keep the amount of other infections down. An unpublished study in our laboratory of a patient with a co-infection with *Bartonella* showed less than expected *Bartonella* infected RBCs in the blood samples. In our view it is not unlikely that co-evolution has taken place regarding the RBC blebbing process in the case of borreliosis.

Lyme disease often persists in spite of repeated medical treatments, and perhaps the most favorable infection sites never become free of bacteria. We think that once infected, the bacterium may easily be established as a kind of life-time “symbiont” in the human body. Depending on the quality of the immune system, several outbreaks of this chronic infection might

develop from time to time during a patient's life-span. The many difficulties in diagnosing *Borrelia* structures in human RBC-samples raise some rather gloomy perspectives for the much used medical practice of blood transfusion. We therefore stress that it is urgent to seriously evaluate the substantial potential for acquiring chronic borreliosis after blood transfusions [25], [26], because the usual methods for detecting *Borrelia* in many cases appears inadequate.

Conclusions

We have developed a laboratory procedure for easy detection of live *Borrelia* spirochaetes and their CWD-forms in human blood from patients with suspected Lyme disease by the use of classic techniques in microscopy. We think it might be of value and assist in the difficult diagnostic work of the disease and help out patients that suffer from chronic health problems without having got a proper diagnosis. We urge that extensive research might be carried out regarding the ecology, life cycle and evolutionary adaptive strategies of this species. Recognition of the importance of CWD-forms and possibly biofilms in persistent *B. burgdorferi* infections (patient "ESH"), should further stimulate pharmaceutical research into new antimicrobial agents that target mechanisms of chronic Lyme disease infection. The ease of this technique and negligible cost makes it useful for any health station provided it has an experienced microbiologist on the staff. If this spirochaete might be transferred during blood transfusions, the potential blood relations between mother and child (prenatally) should also be examined.

Acknowledgement

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Figures

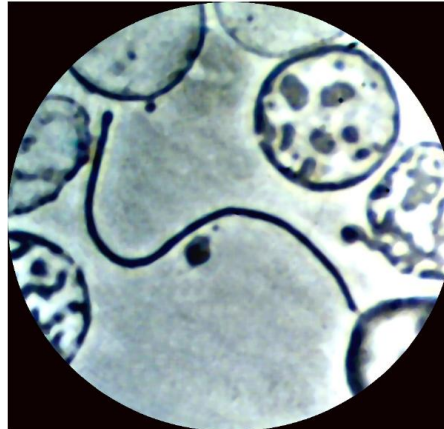


Figure 1. *Borrelia* spirochaete attached to RBC, lower right corner. Note bleb-like swelling in other end. One “pearl” is near center of the image. Structures inside the inflated RBCs are probably infection sites.

Note: Simplified terminology of *Borrelia* stages as seen in the light-microscope:

Motile form:

Spirochaetes appearing as seen in cultured specimens on BSK-medium, several windings of small amplitude along the bacterium. Motile forms are supposed to be present in biofilms and tissues with low oxygen in the body. Rare in blood samples.

Cyst:

Spherical shaped body ca. half the diameter or less of a RBC. Usually non-motile. Inside the protective cover a phase-dense DNA-containing area is seen. Single spirochaetes transform rapidly into cysts when introduced into live blood samples. Cysts are known from our experiments to survive for years even in distilled water.

“Pearl chains”:

Known to be produced by many spirochaetes of different species, also free-living. Are smaller than cysts, originate by transverse constrictions on single spirochaetes. Each pearl contain a complete genome. Single pearls are able to germinate into motile spirochaetes.

Other stages with reduced motility:

A common form in blood has one inflated “bleb” in each end. Between them there are no helices or a few with large amplitude. Some rotation movements appear to exist. Originate either from motile stage in infected tissues or are passively expelled from RBCs. Several kinds appear to exist, many with little resemblance to the motile form.

Blebs and filaments from RBC s:

Blebs, pearl-chains and pearls may be produced by RBCs that do not contain bacteria. Their striking similarity to spirochaete stages are due to a symbiont/coevolution- process between the bacterium and host.

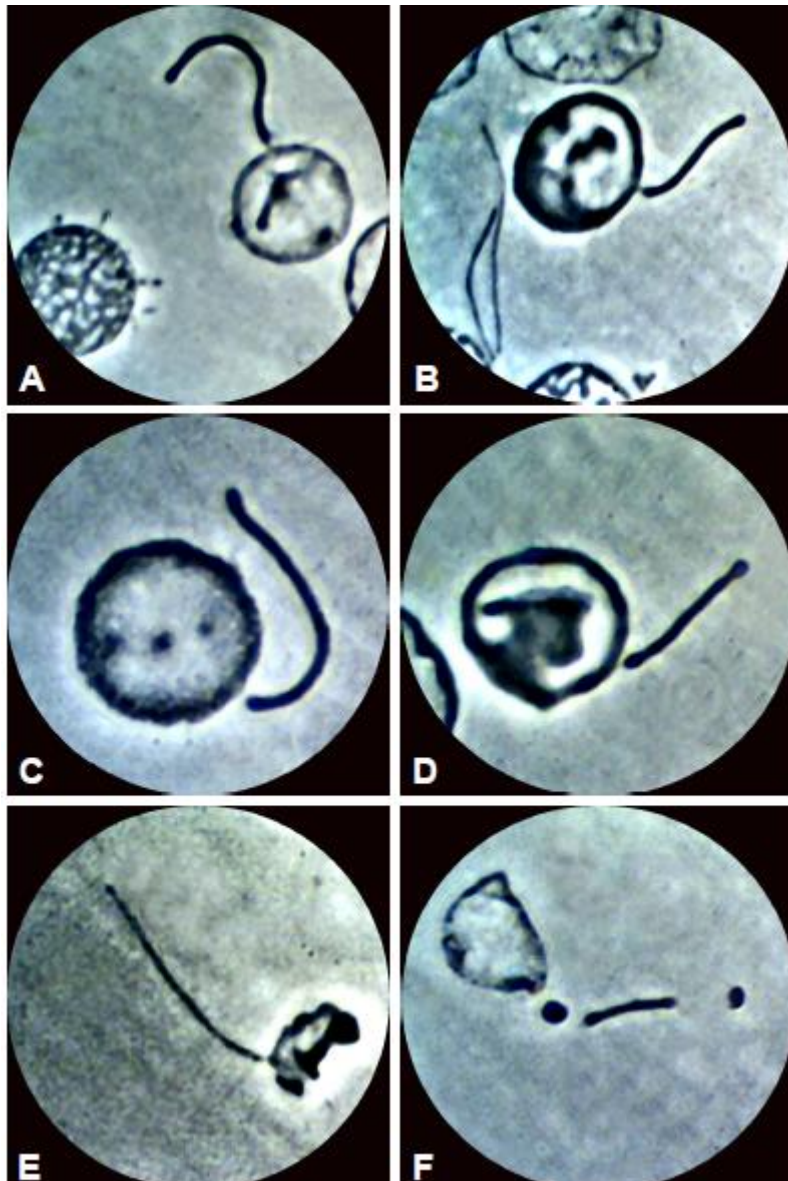


Figure 2. Structural variations of *Borrelia* spirochaete from the same patient (“CS”), (a) a short bent form attached to an RBC still containing a short *Borrelia* inside, (b) an RBC with a straighter *Borrelia* attached, some infective material still inside, two *Borrelia* spirochaetes nearly parallel in lower part of image, (c) *Borrelia* spirochaete detached from the RBC, typical form with a swelling (bleb) at each end, (d) similar nearly straight form detached from decomposing RBC, (e) medium-sized, thin-form *Borrelia*, (f) remnants of cyst, (lower part, one straight and two *Borrelia* spirochaetes in “pearl stage”).

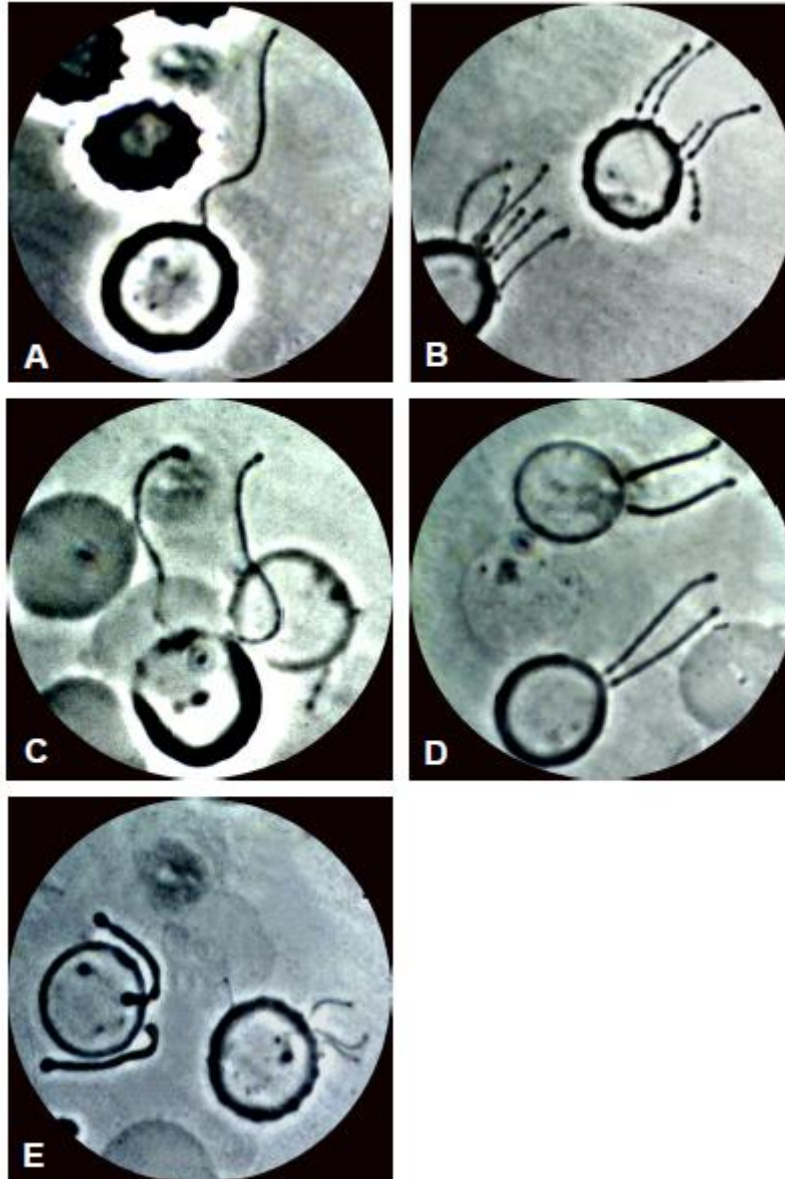


Figure 3. *Borrelia* from another patient (“EN”) with chronic borreliosis diagnosis (confirmed with both DNA and immune techniques). (a) Medium-sized wave-like form attached to RBC, (b) several pearl-stages connected to each of two RBCs, (c-e) pairs of *Borrelia* attached to same RBC s, in (e) part of one spirochaete is situated inside the RBC membrane. This patient had numerous very long and thin wave-shaped *Borrelia* free of the RBC s and an unusual and characteristic diversity of bacterial stages.

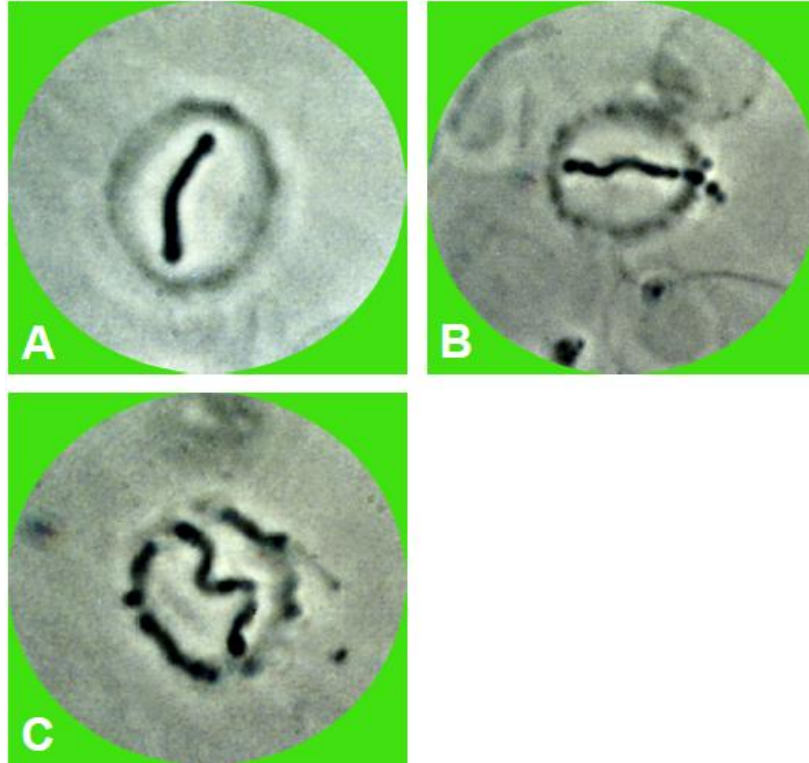


Figure 4. Examples of distinct *Borrelia* spirochaetes inside RBCs from a third patient (“ESH”) (chronic borreliosis diagnosis confirmed with DNA/immune techniques). (a) slightly bent structure with swellings (“blebs”) in each end inside RBC, (b) wave-form partly producing “pearls” leaving the erythrocyte, (c) RBC with a waveform in center, several smaller ones situated close to inner side of RBC membrane.

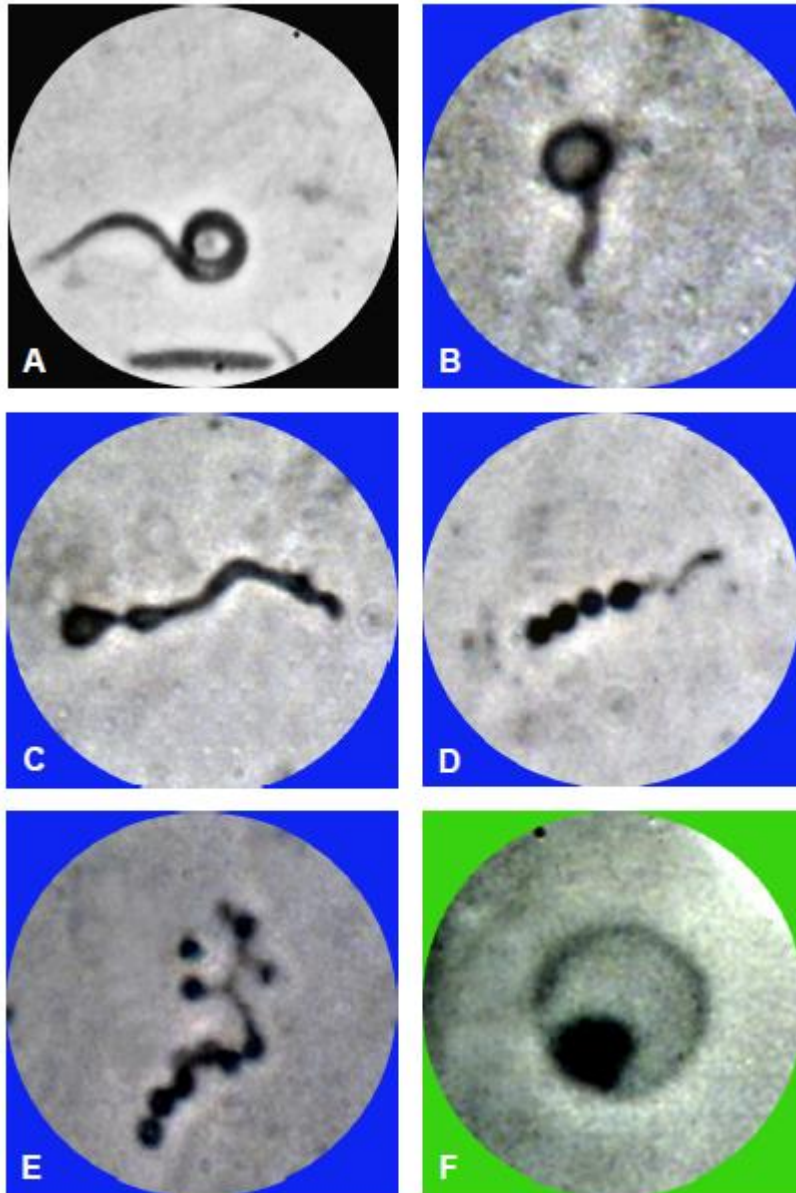


Figure 5. Characteristic stages of spirochaete and *Borrelia* life-cycles: (a) example from a spirochaete inside the gut of the North-American termite *Reticulotermes flavipes* in the process of forming cysts, note coiling up in one end, (b-f) stages from a chronic borreliosis patient (“GR”) (also diagnosed by DNA and immune techniques). Extreme swelling shows details usually not visible by light microscope (LM). (b) germination of “pearl”, tiny *Borrelia* structures appears, (c) somewhat irregular form with large bleb at one end, (d) chain of four “pearls” at one end, thin filament at the other, (e) branched structure with “pearlform”, several varieties occurred, (f) typical cyst, the dark area is the bacterium inside the outer coat. This preparation revealed several forms, some possibly not earlier described.

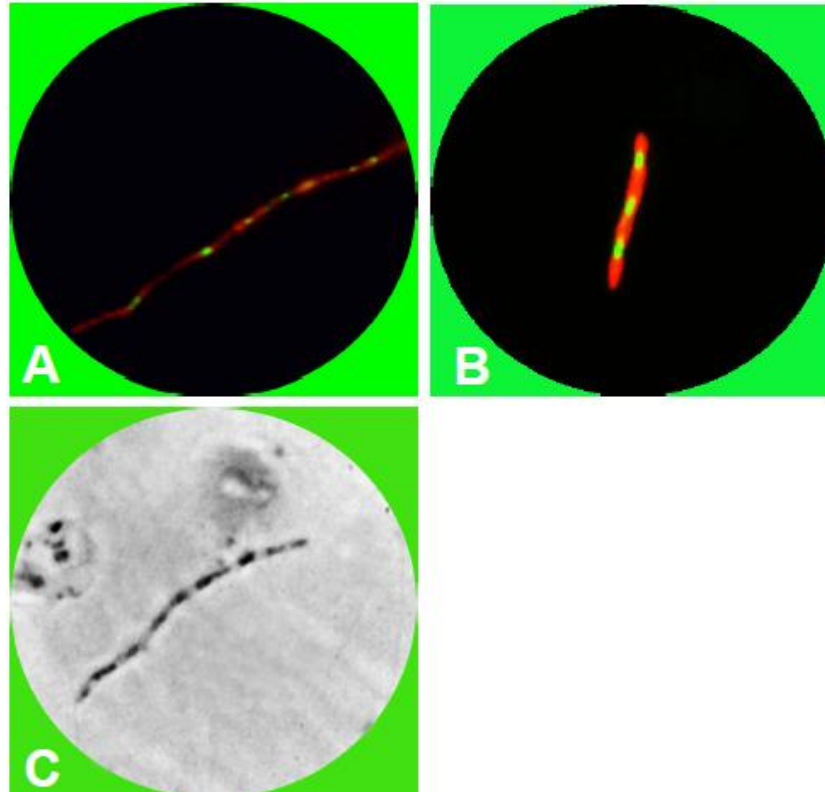


Figure 6. Internal anatomy of *Borrelia* from RBC of patients diagnosed with chronic borreliosis. (a-b) patient (“GR”), (AO) fluorescence staining. Green spots show distribution of the *Borrelia* nucleoids (DNA), orange-red fluorescence represents the bacterial plasma with RNA (c). Nucleoids are also visible by phase-contrast as dark spots (patient “O”). As *Borrelia* spirochaetes divide by transverse divisions, several infective units are produced in each division cycle, compare with reference [29] and [30].