Is sarcoidosis a rickettsiosis?

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LIST OF PAPERS


III. Svendsen CB, Milman N, Andersen CB, Rasmussen EM, Thomsen VO, Krogfelt KA. Sarcoidosis, rickettsiae and mycobacteria: an archival study. Submitted for publication (2)


INTRODUCTION
The first record of the cutaneous lesions seen in sarcoidosis was made by Sir Jonathan Hutchinson in 1877 (5) and later by Ernest Henri Besnier in 1889 (6). In 1899, the Norwegian dermatologist Cæsar Peter Müller Boeck described the cutaneous lesions and named the disease sarcoidosis (7;8). Since then, although our comprehension of the disease has increased, still the disease is far from being completely understood (9).

In 1964, J.G. Scadding suggested three hypotheses about the immunological aberrations of sarcoidosis (10):

1. Sarcoidosis is caused by an etiological agent. The interaction between this agent and the host is the cause of the immunological aberration.

2. Sarcoidosis is to be regarded as a collagenosis or reticulosis in which immunological changes stand at the fore-front.

3. Sarcoidosis only develops in certain individuals previously having immunological aberrations. In these patients sarcoidosis develops as a reaction to a known or unknown agent(s).”

These hypotheses still cover the general perception and were further substantiated in the mid-eighties after a series of studies on the epidemiology of sarcoidosis on the Isle of Man (11-13), as well as by the recent evidence of the existence of “transmissible sarcoidosis” between the footpads of mice (14-16). All of these studies have suggested a transmissible agent to be involved. Despite the fact that new methods have been developed and used during the last 10-15 years (e.g., Matrix-Associated Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF/MS) and proteomics (17;18), 454-sequencing (Svendsen, C.B. et al, ongoing study and (19)), the forty-year-old explanation still holds true.

This thesis will focus on determining whether evidence of previous or current infection with Rickettsia spp. can be found in Danish patients with sarcoidosis in different stages of their disease and whether an infection with Rickettsia spp. can represent an aetiologic factor in the pathogenesis of sarcoidosis.

HYPOTHESIS - THE ASSOCIATION BETWEEN RICKETTSIA AND SARCOIDOSIS
There are several factors necessary in order to be able to demonstrate Rickettsia being involved in the pathogenesis of sarcoidosis in Denmark.

Rickettsiae have to be present as pathogens in Denmark: Four studies have applied rickettsial polymerase chain reactions (PCR) to nucleic acid extractions from Danish ticks with results showing a prevalence of 4.0–13.0% of the investigated ticks; when sequencing of the amplified DNA fragments was performed, Rickettsia helvetica was the agent found in two Swedish patients with sarcoidosis. This virus was cultured in green monkey kidney cells, as rickettsiae, and was then described as myxovirus parainfluenza type 3. The first mention of an association between rickettsial infection and sarcoidosis was in 2002, when Nilsson et al (25) found rickettsial DNA in two Swedish patients with sarcoidosis in a post-mortem investigation. In the same study (25), 26 out of 30 other patients were positive for rickettsial antigens in their tissue samples using an immunohistochemical assay with reactivity to Rickettsia. Planck et al, 2004 (26) found no IgG antibodies directed against Rickettsia in twenty Swedish patients with sarcoidosis. Since
then, Nilsson’s paper (25) has been much criticised by both rickettsiologists and molecular biologists, and the association has remained controversial (27-29).

*Rickettsia* species have been implicated in other chronic diseases, such as chronic perimyocarditis (30), aortic valve disease (31) and chronic fatigue syndrome (32). We hypothesise that a chronic rickettsiosis may also be involved in the initiation and maintenance of the sarcoid immune response due to persistence of bacterial antigen.

### AIMS OF THE THESIS

The aims of this thesis are:

- To develop a novel method for rickettsial diagnostics in Denmark.
- To investigate the association of rickettsial infection and sarcoidosis in Danish patients with sarcoidosis on the basis of:
  - Serological evidence of antibodies to *Rickettsia* in patients with known sarcoidosis.
  - Molecular evidence of nucleic acids in archival tissue biopsy samples from patients with sarcoidosis.
  - Serological, molecular, and clinical evidence of a previous or current rickettsiosis in newly diagnosed patients with sarcoidosis.

### BACKGROUND

**SARCOIDOSIS**

Sarcoidosis is a chronic, granulomatous disease primarily involving the lungs, but with the possibility of affecting every organ in the body (9;33). In Denmark, approximately 400 new cases of sarcoidosis are diagnosed each year, corresponding to an incidence of 7/100 000 person-years; the prevalence in Denmark is approximately 50 000 patients (34). The incidence of the disease is highest around the age of 20-29 years (33). In Scandinavia, there is also a high incidence at the age of ~50 years (35-37). Sarcoidosis in children is rare (0.22–0.27/100 000 children per year) and has a generally benign prognosis where the majority of symptoms resolve completely resulting in normal quality of life in adulthood (38-40).

The common understanding is that sarcoidosis is the result of an abnormal/exaggerated immune response to an external eliciting agent in predisposed individuals (33).

**Clinical aspects of sarcoidosis**

**Symptoms**

Sarcoidosis can have an acute or a chronic presentation. Among Scandinavian patients, the acute debut is often seen in the form of Löfgren’s Syndrome (fever, erythema nodosum (EN), polyarthritis in large joints, and pulmonary bilateral hilar lymphadenitis) (41;42) and can present with or without EN (43;44). The more diffuse, chronic presentation is generally more difficult to recognise as sarcoidosis and thus often presents with signs of organ damage (9;33). The main symptoms of sarcoidosis depend on the organ involved (33;35;36). Most often, at presentation pulmonary symptoms as dyspnoea, dry cough, or chest pain/lingering as well as constitutional symptoms as fatigue, malaise or weight loss are described. In rare cases, the disease can present itself as single organ dysfunction (monocular blindness, facial palsy, renal failure, cardiac arrhythmia, etc.) depending on the affected organ.

The course of the disease depends on gender, age, ethnicity and the affected organs. Generally, spontaneous remissions are seen in two thirds of the patients, while a chronic or progressive disease is seen in 10-30%. In about 15% of the patients with spontaneous remission, some element of organ damage will remain. The course of the disease among African Americans is often more chronic and severe (9;33), while the prognosis in the Japanese population is significantly better than in Caucasian patients (45).

**Diagnostic procedures for sarcoidosis**

Sarcoidosis is regarded as an exclusion diagnosis with the need to rule out the many other reasons for similar symptoms and clinical findings (lymphoma; infections, e.g., mycobacteriosis, toxoplasmosis, histoplasmosis; other interstitial lung diseases and granulomatous disorders, e.g., Wegener’s granulomatosis, allergic alveolitis, and environmental diseases as berylliosis, asbestosis and other pneumoconioses) (9).

After having ruled out the differential diagnoses, the ultimate goal of the diagnosis of sarcoidosis is to obtain a biopsy for (33): 1. Histological confirmation of the disease by demonstrating non-necrotising, epithelioid cell granulomas in the affected tissues. 2. Determine the extent of the disease. 3. Determine whether the disease is stable or progressive.

In other circumstances, the diagnosis is based on typical clinical and/or paraclinical findings such as Löfgren’s Syndrome, Heerfordt’s Syndrome (fever, uveitis, bilateral parotitis, and facial nerve palsy (47)), bilateral hilar lymphadenopathy and elevated disease activity markers as CD4/CD8-ratio, Angiotensin Converting Enzyme (ACE), or soluble Interleukin-2 Receptor (sIL-2R) (33). When evaluating radiographic bilateral hilar lymphadenopathy, Winterbauer et al suggested that if the patient has bilateral hilar lymphadenopathy and a benign disease course on short term, this high probability for sarcoidosis obviates the need for tissue verification (48). No single diagnostic marker has been found and the activity markers ACE and sIL-2R both suffer from low sensitivity and specificity (49).

Even when a biopsy is obtained and it contains non-necrotising, epithelioid cell granulomas, it is important to rule out alternative causes to the granulomas. Thus, special stains for mycobacteria and fungi are performed as well as culture and/or PCR for mycobacteria. In older patients, it must be noted that the localised sarcoid manifestations may be on the basis of malignancy and as such, extra care must be taken (9;33).

Recently, the macrophage enzyme chitotriosidase has emerged as a promising marker of disease activity (50) and there are studies ongoing to establish the use of that marker. The best prognostic marker of sarcoidosis is evidence of the HLA allele HLA-DRB1*0301 that, when found together with Löfgren’s syndrome, predicts a favourable outcome of the disease (51-53).

**Pathogenesis of sarcoidosis**

Recent twin study-based research has suggested that up to two thirds of the susceptibility to sarcoidosis depends upon genetic factors (54). Many different human leukocyte antigen (HLA) alleles (51-53) as well as single genes (e.g., BTN2L (55-60)) have been implicated in sarcoidosis. Recently, we have described lower levels of the pattern recognition molecule ficolin-3 in sarcoidosis patients (61). This could imply that possible...
deficient complement activation takes place in patients with sarcoidosis.

Research has focused on determining which external agent(s) could be responsible for the provocation of the immune response, and the focus still remains on bacteria (62-73) and inorganic substances, e.g. dust and metals (74-79). Various species of propionibacteria and mycobacteria have been extensively studied because of their tendency to cause granuloma formation in human tissue (80-87). With respect to the geographical distribution of sarcoidosis, there is no clear pattern in the epidemiology of sarcoidosis apart from the higher incidence in Scandinavia and among African Americans (9;33).

To date, no firm evidence has been presented towards a specific eliciting agent. The debate has been focused around the evidence of bacteria in the patients/ samples versus the method used for the detection of bacteria.

Genetics in sarcoidosis
Schürmann et al (88) performed the first genetic linkage analysis in sarcoidosis and showed linkage to the entire major histocompatibility complex (MHC) region. Recent reviews by Grünewald (53) and Ianuzzi et al (52) have outlined some of the most important associations.

Specific human leukocyte antigen (HLA) alleles can have impact on both the risk of developing sarcoidosis (HLA-DRB1*01 and HLA-DRB1*04 protect against the disease) and the course of the disease.

<table>
<thead>
<tr>
<th>Agent suspected</th>
<th>Reference</th>
<th>Method used</th>
<th>Conclusion of the study</th>
</tr>
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<tbody>
<tr>
<td>Bacteria</td>
<td></td>
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<tr>
<td>Mycobacteria</td>
<td>Gupta et al (85)</td>
<td>Meta-analysis on PCR studies</td>
<td>Possible association in a subset of patients</td>
</tr>
<tr>
<td>Song et al (18)</td>
<td>Mass spectrometry, Flow cytometry</td>
<td>Possible association</td>
<td></td>
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<tr>
<td>Cell-wall deficient mycobacteria</td>
<td>Cantwell (Journal of Independent Medical Research, JOIMR.org, 2003)</td>
<td>Microscopy</td>
<td>Highly discussed outside the academic press but unlikely association (90)</td>
</tr>
<tr>
<td>Propionibacteria</td>
<td>Eishi et al (81)</td>
<td>PCR on BALF</td>
<td>Propionibacteria are present in higher numbers in sarcoid patients compared to controls</td>
</tr>
<tr>
<td>Ishige et al (83)</td>
<td>PCR on lymph nodes</td>
<td>Propionibacteria are commensal bacteria with no certain pathogenicity</td>
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<tr>
<td>Borrelia spp.</td>
<td>Ishihara et al (68;72)</td>
<td>Serology (ELISA and dot blot)</td>
<td>Higher frequency of antibodies in a subset of sarcoidosis patients</td>
</tr>
<tr>
<td>Chlamydia spp.</td>
<td>Mills et al (91)</td>
<td>PCR</td>
<td>No association</td>
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<td>Virus</td>
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<td>Human herpesvirus-8</td>
<td>DiAlberti et al (92)</td>
<td>Nested PCR</td>
<td>Possible association</td>
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<td>Fungi</td>
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<tr>
<td>Mould/mildew</td>
<td>Newman et al (ACCESS study) (93)</td>
<td>Epidemiological</td>
<td>Increased risk of sarcoidosis</td>
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<td>Inorganic substances</td>
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<tr>
<td>Beryllium</td>
<td>Hardy &amp; Tabershaw (94)</td>
<td>Epidemiological</td>
<td>Separate disease entity (Chronic beryllium disease) (95,96)</td>
</tr>
<tr>
<td>Dust from World Trade Center (September 11, 2001)</td>
<td>Izbicki et al (76)</td>
<td>Epidemiological</td>
<td>Separate disease entity (“sarcoid-like disease”)</td>
</tr>
<tr>
<td>Pesticides/ insecticides</td>
<td>Newman et al (ACCESS study) (93)</td>
<td>Epidemiological</td>
<td>Increased risk of sarcoidosis</td>
</tr>
<tr>
<td>Pine pollen</td>
<td>Cummings et al (97)</td>
<td>Epidemiological</td>
<td>Increased risk of sarcoidosis</td>
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<tr>
<td>Nanoparticles</td>
<td>Heffner (73)</td>
<td>Hypothesis</td>
<td>Unsupported hypothesis</td>
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HLA-DRB1*0301 is strongly linked to a favourable prognosis; also linked with acute onset, fast resolution of disease as well as Löfgren’s syndrome at presentation. Furthermore, specific HLA-DRB1-alleles have different impact on prognosis depending on the phenotype of acute onset disease (43), i.e., among patients with Löfgren’s syndrome, the presence of the HLA-DRB1*03-allele predicted a favourable disease course with resolution within 2 years.

The butyrophilin-like 2 gene (BTNL2) is located on chromosome 6p21 close to the MHC region. Several studies have shown that the single nucleotide polymorphism (rs2076530 G→A) strongly relates to sarcoidosis in different Caucasian populations (Milman, N. et al, unpublished results) but not among African Americans (55-57;60). However, Spagnolo et al (58) showed that the association between sarcoidosis and the BTNL2 A allele could be explained by controlling the statistical analysis for presence of Löfgren’s syndrome and the HLA-DRB1-haplotype. Thus, the proximity of the BTNL2 gene and the MHC region complicates association studies with this particular gene.

Other specific genes of interest are the Toll-like receptor (TLR) genes where expression of TLR2 and TLR4 has been found to be upregulated in sarcoidosis patients (89). Both receptors (TLR2 and TLR4) target ligands originating from bacterial cell walls, i.e., peptidoglycan, lipoteichoic acid and lipopolysaccharide (LPS).

Conclusively, no single gene alterations can explain the disease susceptibility. Nevertheless, several genes involved in the immune system/ pathogen defence system are into play and good prognostic markers exist in the HLA system.

The sarcoid immune response
The hallmark of the sarcoid immune response is the granuloma which is present in the affected tissues. More than 90% of the patients have pulmonary disease and in the lungs, the granulomas are mainly located along the vascular and...
lymphatic bundles but they can be found throughout the pulmonary tissue. The immune response in sarcoidosis is driven primarily by macrophages and monocytes. In the formation of the granulomas, CD4 T-cells differentiate into T-helper cell 1 (Th1+) lymphocytes and secrete interleukin-2 (IL-2) and Interferon-γ (IFNγ) as well as stimulating the macrophage production of Tumor Necrosis Factor α (TNFα); several cytokines are involved (9).

**External eliciting agents**
Several external infectious and non-infectious agents have been suspected of being eliciting agents responsible for the sarcoid immune response. The applied methods for both the diagnosis of sarcoidosis and the detection of antigens have varied, thus making the results difficult to compare.

**Rickettsioses**

**Epidemiology**
Rickettsioses occur throughout the world with preference for the temperate and tropical climate zones. Rickettsiosis was first described in 1873 when early Western inhabitants and physicians referred to the disease as “spotted fever” or “black measles” (99;100). Howard Taylor Ricketts performed a series of experiments between 1906 and 1910 when he found the causative agent of Rocky Mountain Spotted Fever (then called the Spotted Fever of Idaho, figure 1) and demonstrated that it could be transmitted from wood ticks (*Dermacentor andersonii*) to guinea pigs (99). Different rickettsial species give rise to variations of rickettsiosis with the name depending on the species (i.e., Australian Spotted Fever, Queensland Tick Typhus, African Tick Bite Fever, Mediterranean Spotted Fever, Israeli Spotted Fever, Japanese Spotted Fever, and Astrakhan Spotted Fever). The epidemiology of the specific rickettsial diseases depends on properties of the tick vector as each rickettsial species has one or more tick vectors and it is the geographical distribution, seasonal variation and host-seeking behaviour of these vectors that determine the disease (101).

The rickettsial agent *Rickettsia helvetica*, which has been associated with sarcoidosis, has been found ubiquitously in ticks (21;22;102–127) though first isolated in Switzerland in 1979 (106;128). The bacterium is transmitted by a tick, *Ixodes ricinus*, prevalent in Denmark (figure 1) (129;130). The presence of *R. helvetica* in Danish ticks has been confirmed by sequencing of PCR amplicons from four studies with a prevalence ranging from 4 to 13% (20-23).

**Bacteriology**
Rickettsiae are small, gram-stain negative coccobacilli and strictly intracellular (131). The persistence of *Rickettsia* in the environment is dependent on a reservoir host and the bacteria are transmitted by a vector. Both reservoir hosts and vectors vary with different rickettsial species. Spotted Fever Group (SFG) rickettsiae are all transmitted by hard ticks but other vectors include lice, fleas and mites (132). Inside the vector, the rickettsiae are transferred both transstadially from larvae through the nymph stage to the adult tick as well as transovarially to the offspring making it possible for the tick itself to act as a reservoir of the disease (130). The main reservoirs for the SFG rickettsiae in Europe are the tick vectors, dogs, rabbits and rodents (130). Phylogenetically, the genus *Rickettsia* is divided into two major groups: the Spotted Fever Group (SFG) *Rickettsia* and the Typhus Group (TG) *Rickettsia* (131). Members of the SFG are: *R. rickettsii*, *R. conorii*, *R. australis*, *R. honei*, *R. africae*, and *R. helvetica* among others; members of the TG are: *R. typhi* and *R. prowazekii*. The rickettsioses caused by different species of rickettsiae are largely indistinguishable on their clinical presentation, but various diagnostic modalities have allowed the separation into the different groups.

<table>
<thead>
<tr>
<th>Classification of rickettsiae</th>
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<tr>
<td><strong>Kingdom</strong></td>
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<td><strong>Phylum</strong></td>
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<td><strong>Class</strong></td>
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<td><strong>Order</strong></td>
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<tr>
<td><strong>Family</strong></td>
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<td><strong>Genus</strong></td>
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**Figure 1**

**Figure 2**
Clinical aspects of rickettsiosis
Generally, a rickettsial infection will present with: a necrotic wound (“eschar”) at the site of inoculation – the tick bite, a vasculitic rash, fever, lymphadenitis and flu-like symptoms as headache, conjunctivitis and myalgias.

A rickettsiosis caused by *R. helvetica* causes no specific symptoms. Serocconversion towards *R. helvetica* has been described in a patient with flu-like symptoms without rash (108) and *R. helvetica* infection has been associated with chronic fatigue and myalgias in a young man (123).

Rickettsiosis is treated with 2-3 weeks of doxycycline 200mg qd or an equivalent tetracycline. In most rickettsial diseases, the mortality is low (<1%) (28) but recently Brazilian Spotted Fever (with *R. rickettsii*) has been associated with severe disease in Brazil (mortality above 20%) (133).

Chronic cases of infection with *Rickettsia* have rarely been described (134,135), yet post-infectious fatigue has been described in recent case reports by both Nilsson (123) and Watts et al (136). Both reports described young men with serologically confirmed spotted fever group *Rickettsia* infection who suffered from months-lasting myasthenia, myalgia and headache even following appropriate treatment with doxycycline.

Pathology in rickettsiosis
Rickettsiae enter the human body through the tick bite lesion. They are directed towards the blood stream and from there enter endothelial cells by way of receptor-mediated endocytosis (137;138). From the endothelial cells, the rickettsiae can either enter the bloodstream or enter adjacent cells by filopodia. Within the bloodstream, the rickettsiae target circulating endothelial cells and leukocytes.

The hallmarks of the pathology in rickettsiosis are: an increase in vascular permeability; generalized vascular inflammation; oedema; increased leukocyte-endothelium interactions; and release of vasoactive mediators that promote coagulation and pro-inflammatory cytokines (IL-1, IL-6, IL-8). The rash that has given the disease the name “Spotted Fever” is caused by vasculitis in the small cutane vessels.

The primary immune defence against *Rickettsia* depends on the production on IFN-γ by NK cells. Later on in the infection, the humoral immune system takes over and eliminates the infection.

Current diagnostic tests for rickettsiosis
The most recent guidelines for the diagnosis of rickettsiosis (101) state that “Isolation of *rickettsiae* is of great importance, as the ultimate diagnostic goal is recovery of the bacterial agent from a tick or a patient.” However, as rickettsiae are strictly intracellular and classified as Biological Safety Level 3 (BSL-3) agents, the culture can only be performed in and by special laboratories. This limits the use of culture for the diagnostic of rickettsiosis to special circumstances.

On routine basis, the diagnosis is made from a synthesis of the clinical presentation, a medical history with potential or real exposure to ticks, determination of rickettsial antibodies and DNA (101).

Serological methods for detection of rickettsial antibodies
The Weil-Felix test was the first method described for the detection of rickettsial antibodies. It is based on the agglutination of cross-reacting epitopes on whole cell *Proteus vulgaris*OX2 and OX19, *P. mirabilis* OXK and rickettsial antibodies, primarily of IgM type (139). The assay was first described by Weil in 1916 (140), altered and further described by Gilbert in 1935 (141) and Felix in 1944 (142). The method has proven to be of great value in the era before molecular methods became available.

The low sensitivity of the assay and need for increased specificity led to the development of new methods. In 1976, Philip et al (143) published a new method for the detection of antibodies to *Rickettsia*, the immunofluorescence assay (IFA). This was based on the reaction between whole rickettsial cells fixed on a microscope slide. The patient’s serum is then incubated on the slide followed by a second incubation step with a specific fluorescein-marked anti-human IgG/IgM antibody and reading of the slides by epifluorescence microscopy. A semi-quantitative measure of the level of antibodies in the patient can be obtained by serial, two-fold stepwise dilution of the patient serum; positive samples are samples that exhibit fluorescence when diluted above the cut-off of the analysis. This method remains the gold standard for the detection of rickettsial antibodies. Enzyme-linked immunosorbent assay (ELISA) methods where the samples are analysed in microtitre plates have been developed providing for the easier analysis of multiple samples simultaneously (144,145).

A strength of the IFA method is the flexibility of the assay by which the antigen can be substituted to match the specific rickettsiae present in an area; furthermore, several antigens can be combined in each well of the slide allowing for several simultaneous evaluations (termed micro-immunofluorescence). A considerable amount of cross-reactivity is described both between rickettsial species but also to a lesser extent between the two major phylogenetic groups of rickettsiae, the SFG and the TG rickettsiae (139,146-151). Serology can be used to diagnose rickettsioses to the genus level but not to make a specific diagnosis on species level. Furthermore, if the antibody titre is low, a follow-up sample after 2-3 weeks is usually necessary. A four-fold titre increase (e.g., from 256 to 1024) is diagnostic of an ongoing infection (151). If a species level diagnosis is sought from serology, either cross-absorption of antibodies with a panel of different rickettsiae or Western Blotting can be used (101): both methods are labour-intensive and a large amount of serum is needed.

The kinetics of the antibody response also plays a role. Usually IgM and IgG antibodies can be detected 7-15 days after disease onset (101;152). However, in patients with African Tick Bite Fever the median times for the occurrence of IgM and IgG antibodies are 25 and 28 days and administration of doxycycline within a week of disease onset prevents the formation of antibodies (152).

Diagnostic polymerase chain reaction
Molecular methods have been developed to allow a genus- or species-specific diagnosis from a single patient sample (22;101,153-160). When applied on a serum sample or the buffy coat from a plasma sample, rickettsiaemia can be detected shortly after disease onset.

To increase the sensitivity of the PCR, tissue sampling should be done at the primary inoculation eschar or one of the vasculitic lesions: the concentration of rickettsiae in these sites is much higher than in blood (101). The technique is simple, specific, can be used on a variety of sample types, requires a minimal amount of sample and gives an accurate result that can be used for phylogenetic analyses as well as for diagnostic purposes.

A limitation of using PCR as a diagnostic technique is specifically the risk of ampicon carry-over, carrying over DNA fragments previously amplified in the laboratory into the diagnostic sample prior to performing the assay. This yields a
false positive result. To prevent this, strict procedures and quality control must be adhered to (29;161).

**Diagnostic tests that can be used on archival samples**

When performing a diagnosis of rickettsiosis on retrospectively collected samples, the available samples are either frozen serum samples or Formalin-Fixed, Paraffin-Embedded Tissue (FFPET) samples. On the serum samples, IFA can be used with no apparent loss in the diagnostic yield. On the other hand, the FFPET samples can be investigated with either immunohistochemistry or PCR. The immunohistochemistry technique needs specific and well-validated antibodies and has a sensitivity of 50–70% (162-165). Even with specific monoclonal antibodies, cross-reactivity to other bacteria can be seen and in the case of *Rickettsia*, cross-reactions have been described in serum to *Legionella* spp. and *Bartonella* spp. (147;150;166).

The PCR on formalin-fixed samples is limited by the fragmentation and cross-linking of nucleic acids caused by the formalin as well as inhibition of the DNA polymerase enzyme by different compounds in the sample (167;168). When compared to PCR on fresh or frozen tissue, the sensitivity and the diagnostic yield will be lower (168). To circumvent this, a short amplified sequence used in the PCR and specific nucleic acid extraction procedures for FFPET increase sensitivity (168).

**MATERIAL AND METHODS**

**BRONCHOSCOPY**

In study IV (4), tissue biopsy samples were obtained via flexible fibreoptic bronchoscopy (FFB) performed in the Lung Clinic, Copenhagen University Hospital Bispebjerg. The FFB was performed using topical lidocaine as local anaesthetic under sedation with midazolam. Bronchoalveolar lavage (BAL) was performed in the right middle lobe with ~200ml sterile isotonic saline instilled in aliquots of 50ml and recovered with gentle suction. Mucosal Biopsies (MB) were obtained at sites of local inflammation; Transbronchial Lung Biopsies (TBB) were taken guided by the results of the chest radiogram or High-Resolution Computed Tomography (HRCT).

**SAMPLE HANDLING – PROSPECTIVELY COLLECTED SAMPLES**

**Blood samples**

Blood was sampled from patients under aseptic conditions. We sampled blood in EDTA-tubes for plasma and buffy coat separation, uncoated tubes for serology, and heparin-coated tubes for separation of heparinised plasma. After coagulation, the tubes were centrifuged for 8 min at 3500 rpm to precipitate the blood cells.

Serum, EDTA-plasma, and heparinised plasma were separately transferred to 1.8ml cryotubes (Nunc CryoTube™ Vials, 1.8ml, Cat. No. 375418, Nunc A/S, Roskilde, Denmark) and frozen at -80°C.

From the EDTA-tube, the buffy coat was transferred in approximately 1ml plasma to 9ml Red Blood Cell Lysis Solution (Qiagen Danmark, Copenhagen, Denmark). This kit was chosen by Kantsø et al (151). Antigens on the slides were from *R. typhi* and *R. rickettsii*.

**DETERMINATION OF RICKETTSIAL ANTIBODIES**

Serology for Rickettsia was performed using the commercially available immunofluorescence assay (IFA) (IF0100G, Rickettsia IFA IgG, Focus Diagnostics, Inc., Cypress, CA, USA), as described by Kantsø et al (151). Antigens on the slides were from *R. typhi* and *R. rickettsii*.

**FLUORESCENT IN SITU HYBRIDISATION (FISH)**

For the in situ Hybridisation analysis, we developed and used an oligonucleotide probe-based assay specific to the 16S rRNA of *Rickettsia*, as described in paper I (1).

**POLYMERASE CHAIN REACTION (PCR)**

**Nucleic Acid Extraction from Formalin-Fixed, Paraffin-Embedded Tissue (FFPET)**

For the extraction of nucleic acids from the archival tissue samples described in paper III (3), we used the Qiagen FFPE tissue kit (Qiagen Danmark, Copenhagen, Denmark), set up on the automated sample processing device QIAGEN QIACube (Qiagen Danmark, Copenhagen, Denmark). This kit was chosen because of the superior DNA concentration obtained in comparison with other evaluated methods. For the detection of mycobacterial DNA, a combined extraction and strand displacement assay procedure was used, as previously described by Johansen et al (169). The DNA extractions from the mycobacterial assay were also subjected to the real-time PCR for *Rickettsia*.

**Real time PCR**

For the real-time PCR analyses performed in this thesis, we used the genus-level assay described by Stenos et al (170); the assay was modified to run with an internal process control (171) on a different thermocycler (Applied Biosystems ABI 7500) and then re-optimised for reagent concentrations. Bi-annually, we participate in a quality assurance program with Dr Stenos from the Australian Rickettsial Reference Laboratory in Geelong, Australia and Dr Oubaas from PathCare Reference Laboratory in Cape Town, South Africa. To date, we have had...
100% agreement on both sensitivity and specificity in the samples run.

RICKETTSIAL CULTURE

Rickettsiae are small, gram-stain negative cocccobacilli that are strictly intracellular: they must be cultured inside other cells (131). Historically, rickettsiae have been propagated in the yolk sac of hens’ eggs, but for practical purposes cell culture is mostly used today. The preferred cell lines to use for the culture are Vero cells, L929 cells or human embryonic lung (HEL) cells (101;172). Culture from clinical samples can be positive after 1-2 weeks but a culture is not termed negative until 60 days’ incubation.

Culture procedures for positive controls

All work with the cultures to produce positive control samples for PCR and FISH was performed at the BSL-3 laboratory at Statens Serum Institut, Copenhagen, Denmark.

The reference cultures (vials of ~1ml, Australian Rickettsial Reference Laboratory, Geelong, Victoria, Australia) were thawed at 37°C and inoculated in filter-capped culture flasks containing a confluent monolayer of Vero cells (as visualised by inverted phase contrast microscopy) and 9ml sterile RPMI 1640 cell culture medium (1X) with 25mM Hepes (Cat.no. 52400-025, Gibco BRL/Invitrogen corp., USA), modified to contain 10% (v/v) fetal bovine serum, 4.4mg/l amphotericin B, and 263mg/l L-glutamine; the flasks were incubated at 35°C, 5% CO2 for one to two weeks before changing the medium and visually checking for cytopathic effect (CPE), verifying growth using indirect immunofluorescence (IF) with the relevant rickettsial IgG antibodies (RICKETTSIA-POS, Australian Rickettsial Reference Laboratory, Geelong, Victoria, Australia) and FITC-labelled goat anti-human Ig as secondary antibody (Anti-Human IgA+IgG+IgM (H+L) Antibody, FITC labelled, Cat. no.: 02-10-07, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). When monolayers had detached completely because of infection, the cultures were passed on to new flasks in a similar way, 5µm sections were cut off the block and mounted on microscope slides before xylene-based deparaffination as described in paper I (1). For the extraction of rickettsial DNA for positive PCR controls, we spun down the contents of a culture flask at 4,000xg for 10 minutes before heat-inactivating the sample in a water bath at 56°C for 30 minutes. DNA was extracted from this sample using the Qiagen DNA Mini Kit (Qiagen Danmark, Copenhagen, Denmark) according to manufacturer’s specifications.

For the control samples used in the FISH assay, pure bacterial cultures directly from a culture flask were injected into lung tissue from a Specific-Pathogen Free (SPF) swine, fixed for 24-48 hours in 10% (v/v) formalin and subsequently paraffin-embedded and kept in storage for at least 3 months; next, 5µm sections were cut off the block and mounted on microscope slides before xylene-based deparaffinization as described in paper I (1).

Control samples for IF were frozen at -20°C immediately following heat-inactivation or formalin-fixed for 24-48 hours in 10% (v/v) formalin before washing with PBS and store-freezing.

ETHICS AND TRIAL REGISTRATION

We only included samples from patients who were not registered in the Danish National Board of Health’s Tissue Registry (“Vaevsvendelseresgisteret”, http://www.sst.dk/vaev), where it is possible to register a claim of not having samples used for research. All studies were approved by the Regional Medical Ethics Committee (approval no. KF 01 303797) and the Danish Data Protection Agency (approval No. 2006-41-6575). Study IV (4) was duly registered and updated in the ClinicalTrials.gov database (http://www.clinicaltrials.gov) with the Clinical Trials ID NCT00326534 (see appendix II).

DISCUSSION OF METHODS USED IN THIS STUDY

To enhance the validity of our conclusions, we chose to use methods with different characteristics but all targeting Rickettsia. We used a different rickettsial species (Rickettsia australis) as the positive control to prevent contamination of the samples with the species we were particularly interested in (Rickettsia helvetica).

The serological assay chosen to find rickettsial IgG antibodies in paper II (2) and IV (4) is a well-established commercial version of the gold standard immunofluorescence assay. We have validated the assay in the Danish population (151) and to minimize cross-reactivity and enhance specificity, we have reset the cut-off value at a higher level. This essentially eliminates cross-reactions from other antibodies present in the patient’s serum (151). However, when raising the cut-off level, the method loses some sensitivity which is why we also chose to analyse the effect of antibody titres below the cut-off in the samples. We did not analyse the samples for IgM or IgA antibodies.

The real-time PCR developed by Stenos et al (170) used in paper III (3) and IV (4) is well validated in the Australian population and has been used for studies on rickettsial diseases in both Australia (32;170;173;174) and Denmark (22;23). It is able to detect several species of SFG (including Rickettsia helvetica (22;23)) and TG Rickettsia (170) with high sensitivity (170); the PCR does not differentiate between species of Rickettsia.

We have adopted the method for use as a routine PCR in the diagnostic laboratory at Statens Serum Institut and internally validated our setup on clinical samples without problems of false positive samples. Furthermore, we participate in a quality assurance program with two other laboratories. We had perfect agreement between laboratories.

However, the validity of a diagnostic PCR depends greatly on the type and quality of sample used; how it is treated after sampling; the sample preparation; and how nucleic acids are extracted from the sample (161). We have tried to accommodate these issues by selecting a specific DNA extraction procedure and a real-time PCR with a short amplified sequence for the formalin-fixed samples in paper III (3); this should increase the sensitivity compared to conventional PCR. We used a standard method for the DNA extraction from the fresh samples in paper IV (4).

For the visualization of Rickettsia within the tissue samples, we chose to use the FISH technology. This is a very sensitive method with the added benefit of being able to sublocalise the bacteria in the sample (175). From using this, we hoped to determine whether the bacteria were situated in or around the granulomas. An aspect of the FISH analysis that has to be taken into account is that the method targets 16S ribosomal RNA (rRNA) sequences in the bacteria. The content of rRNA varies with growth phase of the bacteria meaning that slower growing bacteria have less rRNA and thus are more difficult to obtain a signal from (176). Resting or inactive bacteria give weak fluorescence signals.
The study was initiated with the intention of additionally using immunohistochemistry as a means for targeting rickettsial antigens within the tissue samples; however, properly validated polyclonal anti-Rickettsia antibodies of good quality were not available through commercial channels and despite several attempts through scientific contacts, we never succeeded in obtaining the relevant antibodies for the immunohistochemistry. The method would have added value to the evaluation of the archival samples, as the protein epitopes targeted by the antibody are kept stable in the formalin-fixed samples and provide for detection of the bacterium after long term storage of the fixed sample.

Because of logistic difficulties with regard to obtaining the biopsy samples in paper IV (4), we did not perform rickettsial culture as part of the diagnostic workup, as immediate processing of the sample is imperative to obtain a culture of Rickettsia (101;172).

**Advantages of rickettsial culture**
- The method is specific.
- Definite causality between infectious agent and symptoms can be obtained following Koch’s postulates (177).
- The isolate can be completely characterised with respect to type, virulence and antibiotic resistance.

**Drawbacks of rickettsial culture**
- The prolonged culture necessary for rickettsiae makes contamination problems a true problem. The addition of antibiotics to the medium can help but not eliminate the problem.
  - Particularly contamination with fungi can be problematic. We experienced extended periods with Aspergillus tereus contamination; this fungus is multi-resistant to antifungals and the addition of amphotericine B to the medium did not eliminate the contamination. We thus had to discard several batches of rickettsial cultures.
  - Up to a third of the cultures are lost to either contamination or lack of growth when the cultures are passed to new flasks (172).
- The culture procedure is time consuming and labour-intensive as the culture flasks need to be evaluated for rickettsial growth or contamination at least once weekly.
- Handling of samples under special conditions is necessary because of the biological safety level (BSL)-3 classification and the fragility of the bacterium (101).

**RESULTS AND DISCUSSION**

**FLUORESCENT IN SITU HYBRIDISATION FOR DETECTION OF RICKETTSIA SPP. – PAPER I, III AND IV (1;3;4)**

**Aim**
The aim of this section was divided in two parts:

First, to develop and validate a novel method for the detection of rickettsial 16S rRNA in archival samples.

Second, to apply the method to tissue biopsy samples from patients with sarcoidosis to determine whether rickettsiae were present in the samples.

**Results**
When validating the method on rickettsial strains embedded in paraffin following formalin fixation, a fluorescent signal was obtained from the rickettsial probe binding to *Rickettsia conorii*, *R. australis*, R. rickettsii, and *R. honei*; there was no signal from *R. typhi* or *R. prowazekii*. From non-rickettsial species, no signal was obtained with the rickettsial probe (1). A Basic Local Alignment Search Tool (BLAST) search using NCBI BLAST (available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed 100% sequence homology between the FISH probe Rick_Cy3 and the 16S rRNA gene from *R. helvetica*.

When combining the results of the rickettsial FISH from papers III and IV, all fluorescence in situ Hybridisation based analyses for the detection of *Rickettsia* were negative (n=114+33=147) in both archival samples and samples from prospectively included patients. In total, seventy-two samples were tested with the eubacterial probe; results are summarized in table 3. When excluding the mycobacterial samples, there was no significant difference in the detection of eubacteria among sarcoidosis patients and control patients (Fisher’s Exact Test, p=0.17).

<table>
<thead>
<tr>
<th></th>
<th>Sarcoiosis</th>
<th>Control</th>
<th>Mycobacteriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos</td>
<td>n=13+25=38</td>
<td>n=18+5=27</td>
<td>n=9+2=11</td>
</tr>
<tr>
<td>Neg</td>
<td>n=27+40=67</td>
<td>n=26+19=45</td>
<td>n=14+3=17</td>
</tr>
<tr>
<td>Fisher’s Exact test</td>
<td>0.047*</td>
<td>0.17**</td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**
For the detection of *Rickettsia* in archival samples, we developed an assay based on fluorescence in situ hybridisation. The method showed specific hybridisation to rickettsial species within the Spotted Fever Group but did not detect rickettsiae from the Typhus Group. The rickettsial probe did not give any signal from non-rickettsial species, including *Coxiella burnetii*.

When using this assay on both the archival samples from paper III (3) and the prospectively included samples from paper IV (4), no patients had evidence of *Rickettsia* in their tissue. We have no reason to believe that insufficiency of our assay was the reason for the lack of detection of Rickettsia in the samples as all our positive and negative control samples for the assay performed as expected. However, Amann et al (176) adequately pointed out the limitations of the FISH technique in that it targets the 16S RNA sequences: hence, slowly-growing or resting bacteria are not easily identified, as the amount of RNA in bacteria depends on the growth phase (176). Should the bacteria present in the tissue samples therefore be resting or slowly growing, our assay could possibly not have detected them.

There was a striking difference in the frequency of obtaining a positive signal from the eubacterial probe in the two populations. In paper III (3), 24 of the 36 samples evaluated contained bacteria whereas only 2 of the 33 samples from paper IV (4) contained bacteria.

Apart from 12 samples, the samples in paper III (3) (n=114) were all mediastinal lymph node biopsies obtained by mediastinoscopy while the samples in paper IV (4) were...
obtained by FFB. Should this have had an effect on the difference, we would have expected the bronchoscopic samples from paper IV (4) to have a higher frequency of bacteria, as the bronchoscope is introduced through the nasopharyngeal region where a rich natural flora is present (179).

It is probable that the samples from paper III (3) were contaminated during storage as they had been kept in the archives for longer than the samples in paper IV (4).

In conclusion, we have developed a novel assay for the detection of rickettsiae in archival samples. When using this assay to evaluate the primary hypothesis of the study, we could not find evidence of rickettsiae in any of the tissue samples. Thus, the FISH analysis cannot support the hypothesis.

In contrast, we did find several of the samples in the archival study positive for bacteria of unclassified nature. There was a trend in the subset of the data pointing towards more bacteria being present in the sarcoidosis patients than in the control patients. This observation lends support to the hypothesis of bacteria being involved in the sarcoid pathogenesis. We are currently using laser capture microdissection (180) in combination with 16S rRNA gene based eubacterial PCR on the fluorescently labelled bacteria in order to obtain an identification of the species to further comment on whether they are potential pathogens or merely the result of sample contamination.

DETERMINATION OF RICKETTSIAL ANTIBODIES IN DANISH PATIENTS WITH SARCOIDOSIS – PAPER II (2) AND IV (4)

Aim
The aim of this part of the thesis was to detect IgG antibodies originating from a previous or ongoing rickettsial infection in patients with sarcoidosis.

In addition, plasma samples were screened for antinuclear antibodies (ANA) because the presence of ANA may cause unspecific reactions in Vero cell based immunofluorescence assays (181).

Results
Full results are summarised in table 4. The prevalence of any reactivity in the rickettsial antibody assay in patients with sarcoidosis 15/73 (20.5%) was not significantly different from the prevalence in the control patients 14/61 (22.9%) (Fisher’s Exact Test, p=0.84).

The prevalence of antinuclear antibodies was 6/72 (8%) among the sarcoidosis patients and 4/56 (7%) among the control patients (Fisher’s Exact Test, p=0.43). The patterns of antinuclear antibodies found in control patients were: homogenous (n=1), fine-speckled (n=1), mitotic spindle apparatus (n=1) and coarse-speckled (n=1) pattern; among sarcoidosis patients: homogenous (n=1), fine-speckled (n=1), mitotic spindle apparatus (n=1), centromere (n=1), nuclear dot (n=2) and nucleolar (n=1) pattern. One sarcoidosis patient had both nuclear dot antibodies and homogenous antibodies (see figure 3, courtesy of Mimi Høier-Madsen, SSI).

Table 4
Antibodies in the study population

<table>
<thead>
<tr>
<th>% (n)</th>
<th>Sarcoidosis patients</th>
<th>Control patients</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive for rickettsial IgG antibodies (any titre, any antigen)</td>
<td>21% (15/73)</td>
<td>23% (14/61)</td>
<td>0.84</td>
</tr>
<tr>
<td>Positive for rickettsial IgG antibodies (Titre ≥ 512)</td>
<td>1% (1/73)</td>
<td>0% (0/61)</td>
<td>-</td>
</tr>
<tr>
<td>Positive for antinuclear IgG antibodies (any pattern) (Titre ≥ 160)</td>
<td>8% (6/72)</td>
<td>7% (4/56)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Thirty-nine patients (52%, n=75) were under or had received treatment with prednisolone in the three months preceding the blood sample; the patients had been treated with median 20 mg (2.5–50) prednisolone/day) for median 6 (1–300) months. Plasma total IgG concentration in patients (n=38) treated with prednisolone (median 10.5 g/L, interquartile range (IQR) 8.0–13.0) was significantly lower than in patients (n=28) who did not receive prednisolone (median 13.2 g/L, IQR 11.3–14.5) (Mann-Whitney U test, p=0.004). Where information was available among sarcoidosis patients and control patients (n=83), there was no effect of prednisolone treatment upon the reactivity in the serological assay (table 5, Fisher’s exact test, p=0.2).

Table 5
Reactivity in the rickettsial immunofluorescence assay by prednisolone treatment.

<table>
<thead>
<tr>
<th>Prednisolone treatment</th>
<th>No reactivity</th>
<th>Reactivity</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>30</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td>Yes</td>
<td>32</td>
<td>7</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>21</td>
<td>83</td>
</tr>
</tbody>
</table>

p=0.2; Fisher’s Exact Test

Figure 3
Discussion
We have shown antibody reactivity among sarcoidosis patients and control patients to antigens from rickettsial species. There was no significant difference between the reactivity in the serum of sarcoidosis patients and control patients and we found no effect of prednisolone treatment on the level of rickettsial antibodies. Only one patient had IgG antibodies to *Rickettsia* above the cut-off of the assay.

Our results from the serological assay are in compliance with the results of Planck et al (26) who also found no antibodies in 20 Swedish sarcoidosis patients.

Reasons for the lack of rickettsial antibodies in the sarcoidosis patients could be several:

A. That a rickettsial infection may not be involved in sarcoidosis.

B. That the immune reaction towards *Rickettsia helvetica* does not give cross-reactivity to the immunodominant epitopes from *R. rickettsii* or *R. typhi* on the IFA used.

We believe our assay should have been able to detect antibodies from a rickettsiosis with *R. helvetica*. This is based on the extensive cross-reactivity between different species within the SFG (182). However, Elfving et al (118) suggested the major immune reactivity in *R. helvetica* to be directed at surface protein antigens rather than the reactivity of other rickettsial species that is principally directed at lipopolysaccharide (LPS) (183). Hence, a weakened antibody response towards the antigens used in the IFA could mean decreased sensitivity. Another species shown to cause reactivity to LPS in only 60% of patients is *R. africae*, as shown by Jensenius et al (184). Hajem et al (185) have described an immunoreactive protein (35kDa, RC0799-like protein) hitherto only seen in *R. helvetica*. If the dominant immune response in a rickettsiosis with *R. helvetica* was due to this protein, conventional whole cell rickettsial assays could have problems detecting the antibodies.

C. That immunosuppressive treatment with prednisolone attenuated the immune reactivity towards *Rickettsia*.

The patients treated with prednisolone had a lower level of total IgG antibodies in the blood but no significantly altered reactivity in the rickettsial IFA (table 5) thus making this explanation unlikely. Furthermore, only few of the patients in the prospective trial (4) had been treated with immunosuppressives.

D. That the humoral immune response to the bacterium had disappeared in spite of persisting bacterial antigen causing granulomas.

A similar theory has recently been described with the related pathogen *Coxiella burnetii* by Lockhart, M. et al who presented a case story on persistent Q-fever demonstrated with PCR, with no evidence of antibodies to *Coxiella* using a *Coxiella*-specific IFA (Presentation at 5th International Conference on Rickettsiae and Rickettsial Diseases, Marseille, May 2008).

We would normally expect antibodies to be present in a persisting infection. However, if the infection was cleared by the immune system, and the sarcoid response was because of the remaining bacterial residues, we would not necessarily expect an antibody response to be sustained (186). If the immune response to a chronic infection with *Rickettsia* was predominantly cellular, as demonstrated in murine models of early infection (187), the antibody response could also be weaker. Weak antibody responses have been observed in infection with *R. africae* and *R. slovaca* (152;188). Furthermore, the sarcoid granuloma could represent the inflammatory response to *Rickettsia* in a patient incapable of producing a sufficient antibody response, as previously proposed by Reich (189).

In conclusion, we cannot conclude from the serological data alone that a rickettsial infection is not involved in the pathogenesis of sarcoidosis, but our results do not support the primary hypothesis of the study.

POLYMERASE CHAIN REACTION-BASED DETECTION OF RICKETTSIA IN FORMALIN-FIXED, PARAFFIN EMBEDDED TISSUE – PAPER III (3) AND IV (4)

Aim
To detect DNA from rickettsiae in tissue biopsy samples from patients with sarcoidosis using PCR.

Results
No samples from either the archival study described in paper III (3) or the prospective study described in paper IV (4) contained evidence of rickettsial nucleic acids by PCR. All controls performed as expected.

Discussion
The PCR assay did not show any sign of rickettsial infection among sarcoidosis patients or control patients and cannot support the hypothesis of a rickettsial agent being involved in the sarcoid pathogenesis.

Several factors can have influenced the lack of detection of nucleic acids from *Rickettsia* in the samples. First, the biopsies could have been obtained outside a site of localized rickettsial infection. However, this is unlikely as the biopsies from the sarcoidosis patients all contained granulomas and we would expect the granulomas to be located around the antigen responsible for the immune response. If samples containing nucleic acids are kept at room temperature, endogenous nucleases will be active and degrade the DNA in the sample. Cushwa et al (190) have shown a clear dependency on DNA yield of temperature and storage time.

All samples from the prospective study (4) were readily transferred to -30°C and from there to -80°C to inhibit the activity of DNAase enzymes in the tissue; immediately after thawing, DNA was extracted for the PCR assay and stored again at -20°C.

The archival samples from paper III (3) had been kept in storage following routine departmental procedures and DNA extracted using a specific method. Though the samples had been in the archive for up to 10 years, we were still able to detect mycobacterial DNA in some of the samples, meaning that PCR inhibiting compounds cannot have been present in significant amounts.

The PCR method used to detect *Rickettsia* in the samples has been used in several other studies and we have also detected *R. helvetica* in Danish ticks using the method (22;23). Furthermore, we participate in an annual quality assurance program with the rickettsial PCR with excellent results.

The limitations in using the PCR method mainly relates to the archival samples, where we cannot claim that formalin-induced DNA damage did not have a deleterious effect on the detection limit of the PCR. However, the negative samples from the prospective study (4) and the positive detection of
CONCLUSION AND FUTURE PERSPECTIVES

When summing up the existing evidence for the association between *Rickettsia* and sarcoidosis (2-4;25;26), the support for the association is very limited (25). By combining several diagnostic modalities for the detection of rickettsiae, we have increased the validity of our negative findings. We could not detect rickettsial IgG antibodies, rickettsial DNA or rickettsial 16S rRNA in any of the samples. Furthermore, from the patients in whom information was available, there was no significant difference in the frequency of reporting a tick bite (4).

All of our assays were specific to the genus level and were set up not to distinguish between species. In the validation of our assays, we included samples containing either *Rickettsia* spp. DNA or whole cell *Rickettsia* as appropriate. Our assays have detected *R. helvetica* in previous studies (22;23) as well as *R. africae* in several recently diagnosed patients with African Tick Bite Fever returned from South Africa (Svendsen, C.B. et al, unpublished data). The serological assay has been used for the detection of antibodies originating from rickettsioses with *R. typhi* and *R. a. rickettsii*. The negative outcome of the specific *R. helvetica* assay in paper II (2) was probably related to a weaker antigen, producing fewer cross-reactions than the *R. rickettsii* antigen from the Focus kit. We have presented data on the difference in quality of the antigens between kits in a recent paper (151). Because of the wide cross-reactivity of the IFA among the SFG rickettsiae, the serological assay used should detect antigens from rickettsiosis with *R. helvetica* even though it uses *R. rickettsii* as the SFG antigen (2;28;151). The negative outcome of the specific *R. helvetica* assay in paper II (2) was probably related to a weaker antigen, producing fewer cross-reactions than the *R. rickettsii* antigen from the Focus kit. We have presented data on the difference in quality of the antigens between kits in a recent paper (151). The granuloma is the hallmark lesion of sarcoidosis and to our knowledge, granulomas have never been described in rickettsiosis, only in infections with the related pathogens *Coxiella* (191) and *Bartonella* (192).

In Scandinavia, predominantly imported cases of rickettsiosis have been reported (193-196); however, Nilsson (123) has described a single case of infection with *R. helvetica* in a young Swedish man. Another recent publication from Sweden (118) showed a seroprevalence of antibodies to *R. helvetica* in tick-bitten patients to be 0.6 to 4.4% depending on history of previous tick bite, highest in patients with *Borrelia* antibodies and lowest in healthy blood donors. To this effect, rickettsial disease is proven to be present in Scandinavia but both Sweden and Denmark (151) can be classified as low-prevalence countries with regard to rickettsiosis. This contrasts with the relatively high incidence of sarcoidosis in the Scandinavian countries, as described by several authors (9;33;35;37;98).

The incidence of neuroborreliosis has been shown by Jensen et al (129) to be a valid indicator of the tick density/ risk of tick bite in Denmark. When comparing these data to the incidence of sarcoidosis in Denmark from Byg et al (34), a paucity of neuroborreliosis cases West of the late Weichselian ice front in Western Jutland converts to a strong negative correlation with the incidence of sarcoidosis (Spearman’s rho = -0.78, p=0.003, n=12; table 6 and figure 4). Denmark has a clear East to West gradient with regard to the sarcoidosis incidence in the opposite direction of the distribution of neuroborreliosis. The interpretation of this would be that tick bite or any agent transmitted via a tick bite does not increase the risk of sarcoidosis.

### Table 6

<table>
<thead>
<tr>
<th>Region (Danish: amt)</th>
<th>Cases of neuroborreliosis per 1,000 inhabitants, 1993-1995 (129)</th>
<th>Incidence of sarcoidosis per 100,000 person-years, 1990-1994 (34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bornholm</td>
<td>0.080</td>
<td>2.6</td>
</tr>
<tr>
<td>Vestsjaelland</td>
<td>0.051</td>
<td>4.0</td>
</tr>
<tr>
<td>Storstroem</td>
<td>0.048</td>
<td>7.6</td>
</tr>
<tr>
<td>Fyn</td>
<td>0.039</td>
<td>7.0</td>
</tr>
<tr>
<td>Roskilde</td>
<td>0.037</td>
<td>3.4</td>
</tr>
<tr>
<td>Frederiksberg</td>
<td>0.034</td>
<td>4.8</td>
</tr>
<tr>
<td>Vejle</td>
<td>0.024</td>
<td>4.9</td>
</tr>
<tr>
<td>Copenhagen</td>
<td>0.021</td>
<td>5.3</td>
</tr>
<tr>
<td>Ribe</td>
<td>0.020</td>
<td>8.6</td>
</tr>
<tr>
<td>Viborg</td>
<td>0.017</td>
<td>7.1</td>
</tr>
<tr>
<td>Ringkoebing</td>
<td>0.008</td>
<td>8.6</td>
</tr>
<tr>
<td>Nordjylland</td>
<td>0.008</td>
<td>7.9</td>
</tr>
<tr>
<td>Soenderjylland</td>
<td>n/a</td>
<td>7.0</td>
</tr>
<tr>
<td>Aarhus</td>
<td>n/a</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Spearman’s rho = -0.78, p=0.003, n=12

Conclusively, our study cannot support that a tick-borne infection, in particular rickettsiosis, should be involved in the pathogenesis of sarcoidosis.
primers amplifies a shorter fragment of the first amplicon. This increases sensitivity but the PCR tubes need to be re-opened in the primary laboratory to run the second PCR. Likewise, a “suicide PCR” has been described for rickettsial diagnostics enabling a high sensitivity but precluding the use of the same primer set again in the lab because of contamination risk (197).

We used separate rooms in separate buildings for setting up PCR reactions and running the PCRs (22): this minimises the risk of amplicon carry-over.

The immunohistochemical assay in Nilsson’s study (25) is based on the cross-reaction between rickettsial antibodies and the Proteus antigen also used in the Weil-Felix reaction (140). In Nilsson’s setup, anti-Proteus vulgaris OX19 antibodies were incubated on the samples reacting with epitopes from antigens from what is described as “Rickettsia-like organisms”. Strengthening the results from the primary immunohistochemical analysis in Nilsson’s study (25), all positive samples were confirmed using specific rickettsial mono- or polyclonal antibodies and the PCR results from the autopsy cases described were confirmed by immunohistochemistry and electron microscopy. Any potential cross-reactivity of Nilsson’s antibodies was not described in his paper (25) but rickettsial antibodies are known to cross-react with both Legionella pneumophila serogroup 1, Legionella bozemanii (147) and potentially Bartonella species (166): the rickettsial FISH probe did not bind to any of these three species of bacteria (1).

We initiated the present study based on positive results from a pilot study using the same Proteus-antibodies as Nilsson et al (25). In that pilot study, we identified Rickettsia-like organisms in 7 out of 13 samples using Proteus-antibody based immunohistochemistry and in 6 out of 10 samples using rickettsial FISH. The samples originated from the same archive as described in paper III (3). In the present study, we did not repeat the immunohistochemistry because we could not obtain specific rickettsial antibodies to confirm possible positive samples.

No other studies have used this particular immunohistochemical technique to diagnose a rickettsiosis and the method has been criticised by prominent rickettsiologists (27;28).

There is increasing evidence towards one or more infectious agent(s) being responsible for eliciting the sarcoid immune response. These agent(s) could vary depending on geography and different antigens could be eliciting the sarcoid response in different populations.

The search for the culprit pathogen(s) continues with the focus still largely being on mycobacteria. With 26 ongoing clinical trials (ClinicalTrials.gov search “sarcoidosis”, April 23, 2009) and ~19,000 publications on sarcoidosis in PubMed alone, the disease is getting increasingly well-characterised (PubMed search “sarcoidosis” [mesh], April 23, 2009) and new approaches such as lymphocyte stimulation assays, proteomics, lipidomics and sensitive molecular methods as 454-sequencing will hopefully provide augmented insight into the sarcoid aetiology.

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>BSL-3</td>
<td>Biosafety Level 3</td>
</tr>
<tr>
<td>BTNL2</td>
<td>Butyrophilin-like 2</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathogenic Effect</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>FFB</td>
<td>Flexible Fibreoptic Bronchoscopy</td>
</tr>
<tr>
<td>FFPET</td>
<td>Formalin-Fixed, Paraffin-Embedded Tissue</td>
</tr>
<tr>
<td>FFT</td>
<td>Fresh, Frozen Tissue</td>
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<tr>
<td>FISH</td>
<td>Fluorescent In Situ Hybridisation</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>IFA</td>
<td>Immunofluorescence Assay</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>INFγ</td>
<td>Interferon-γ</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-associated laser desorption/ionization-time of flight mass spectrometry</td>
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<tr>
<td>MB</td>
<td>Mucosal Biopsy</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>R.</td>
<td>Rickettsia</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>sIL-2R</td>
<td>Soluble interleukin-2 receptor</td>
</tr>
<tr>
<td>SFG</td>
<td>Spotted Fever Group</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific-Pathogen Free</td>
</tr>
<tr>
<td>Th1+</td>
<td>T-helper cell type 1</td>
</tr>
<tr>
<td>Th2+</td>
<td>T-helper cell type</td>
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<tr>
<td>TG</td>
<td>Typhus Group</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TBB</td>
<td>Transbronchial Lung Biopsy</td>
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</table>

SUMMARY

The pathogenesis of sarcoidosis is still largely unknown. The generally accepted theory is that genetically predisposed individuals develop the sarcoid disease reaction as a response to one or more unknown antigen(s). A single study by Nilsson et al has related the development of sarcoidosis to an infection with *Rickettsia helvetica*.

The aim of this thesis was to investigate whether a rickettsial infection is involved in the pathogenesis of sarcoidosis. We used different microbiological methods as serology, polymerase chain reaction, and fluorescent in situ hybridization (FISH) on samples from patients with sarcoidosis and control patients. The thesis compiles the results from four separate studies: The second paper describes a serological survey in historical patient sera. None of the results from the studies supported the hypothesis of *Rickettsia* being involved in the pathogenesis of sarcoidosis.

In conclusion, we could not find evidence to support the primary hypothesis of the study, that a rickettsial infection should be involved in the pathogenesis of sarcoidosis.

REFERENCES

6. Besnier E. Lupus pernio de la face; synovites fongueuses (scrofulotuberculeuses) symétriques des extrémités superieures. Annales


11. Lægevidenskaben, Kristiania (Oslo) 1899;60:1321-34.


83. Milman N. From mycobacteria to sarcoidosis—is the gate still open? Respiration 2006;73(1):1-5.


