Journal of Spirochetal and Tick-borne Diseases

Fall/Winter 2000 Volume 7

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Seroepidemiology of the Rickettsioses, Human Granulocytic Ehrlichiosis, Lyme Disease, Q Fever, and Tularemia in Forestry Workers in Tuscany, Italy

Donatella Aquilini, MD; P. Parola, MD†; E. Salvo†; and A. Paladini, MD**

ABSTRACT

The prevalence of antibodies reactive with tick-borne bacterial pathogens was investigated using a microimmunofluorescence test on human sera obtained from 507 forestry workers of Tuscany, Italy. Antibodies reactive at titer ≥1:50 were found against *Rickettsia conorii* (2.4%), *Rickettsia slovaca* (3.4%), *Rickettsia helvetica* (1.0%), *Rickettsia mongolotimonae* (3.0%), *Rickettsia massiliae* (2.8%), *Coxiella burnetii* (24.9%), *Borrelia burgdorferi* (17.4%), and *Francisella tularensis* (2.6%) but not against the human granulocytic ehrlichiosis (HGE) agent. These results confirm the presence of tick-borne bacterial diseases in this population and open up important prospects of study and work in this area. Acute specific diseases, particularly emerging rickettsioses, have yet to be identified.

Key words: Tuscany, bacterial pathogen, Lyme disease, Q Fever, rickettsia

INTRODUCTION

Spotted fever group rickettsiae are gram-negative intracellular bacilli associated with arthropods. Ticks are their main reservoirs and vectors. Five human rickettsioses have been reported in western Europe. *Rickettsia conorii*, the agent of Mediterranean spotted fever ("boutonneuse fever") is the most frequently encountered spotted fever agent in the Mediterranean area and is transmitted through the bite of the dog brown tick, *Rhipicephalus sanguineus*.[1] In 1910, the disease was described with the clinical characteristics of fever, headache, rash, and eschar formation. Although until recently *R conorii* was thought to be the only pathogenic rickettsia, the study of atypical cases in endemic areas led to the description of new clinical syndromes caused by emerging pathogens. Israeli spotted fever is caused by a rickettsia closely related to *Rickettsia conorii*. The inoculation eschar is lacking and the disease may be severe. It has been known in Israel since 1974 and was recently identified in Sicily and Portugal.[2] *Rickettsia mongolotimonae* was isolated in 1991 from *Hyalomma asiaticum* ticks collected in Mongolia. In March 1996, it was isolated in a patient with mild spotted fever in France and a second case was described in 1998.[3,4] The vector has not been determined in this area. In 1997, the first documented case of infection caused by *Rickettsia slovaca* was reported.[5,6] The disease is transmitted by *Dermacentor marginatus* and *Dermacentor reticulatus* and clinical characteristics include eschar formation (particularly on the head) and cervical lymphadenopathy. More recently, *Rickettsia helvetica* was isolated in Switzerland,[7] France,[8] and Sweden[9] from *Ixodes ricinus* ticks and has been recently associated with perimyocarditis in man.[10] In addition to these pathogens, some rickettsiae of unknown pathogenicity including *Rickettsia rhipicephali, Rickettsia massiliae,* and closely related strains have been isolated only in ticks in France, Spain, Portugal, and Greece.[1]

The other human bacterial diseases transmitted by Ixodids (hard ticks) in Europe include human granulocytic ehrlichiosis, Lyme disease, tularemia, and Query (Q) fever. Human granulocytic ehrlichiosis (HGE) is a potentially severe undifferentiated febrile illness that was first described in the USA in 1994[11] and has subsequently

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Figure 1. Tuscany map and geographical distribution of studied areas.

been shown to occur in Europe.[12-15] The causative organism is currently known as the HGE agent and is very closely related to *Ehrlichia equi* and *Ehrlichia phagocytophila* (pathogens of horses and ruminants, respectively). *Ixodes ricinus* ticks have been suggested as vectors in Europe. Lyme disease is also transmitted by *Ixodes ricinus* in western Europe. It is caused by three species of the *Borrelia burgdorferi* complex. *Borrelia afzelii* is associated with dermatological forms and a milder disease than that caused by *Borrelia burgdorferi* sensu stricto (ss).[16,17] Infections with *Borrelia garinii* are more frequently associated with neurological abnormalities while those with *B burgdorferi* ss are more often associated with rheumatological disorders.[16,17] Q fever is an ubiquitous zoonotic disease caused by *Coxiella burnetii*. The disease is usually acquired by the ingestion or inhalation of virulent organisms from infected mammals or their products. Although the organism has been found to infect numerous ticks, their role in human infections is probably minimal.[18] In the same area, tularemia is caused by *Francisella tularensis* biogroup *holartica* and tick vectors include *Ixodes ricinus* and *Dermacentor sp.*[19,20]

The aim of the present study was to determine the seroprevalence of antibodies against spotted fever rickettsiae (*R conorii, R slovaca, R mongolotimonae, R massiliae, R helvetica*), the HGE agent, *Borrelia burgdorferi, Coxiella burnetii,* and *Francisella tularensis* in forestry workers in Tuscany, Italy.

MATERIAL AND METHODS

Study Area

The investigation was conducted in 10 different districts in Tuscany: (i) Alberese and San Rossore on the coast of the Tyrrhenian sea, (ii) Arcidosso, Acquerino, Barga, Fivizzano, San Marcello Pistoiese, and Villafranca in the hills, and (iii) Abbadia San Salvatore and Abetone in the mountains (**Figure 1**).

Serum Samples and Serology

Serum samples were obtained from 507 forestry workers over a period between 1988 and 1991. Participants were asked to answer a questionnaire providing information about age, sex, place of residence, time of occupational exposure, previous tick bites, contact with wild and domestic animals, outdoor activities (fishing, hunting, and searching for mushrooms), consumption of raw milk, and previous diseases related to the investigated agents. Sera were collected and stored at -20°C until serology was performed at the National Reference Centre for Rickettsioses in Marseille, France (Professor Didier Raoult). All the sera were tested by immunofluorescence, as previously described.[21] Determination of antibodies was carried out at 3 two-fold dilutions 1:25, 1:50, and 1:100. A dilution of 1:50 was considered positive.

Statistical Analysis

Statistical testing was performed by Statistics for Windows program, Release 4.5 (Stat Soft Inc). The Chi2 test or the Fisher's exact test was used, as appropriate, to evaluate the relationship between seroprevalence and risk factors. The relationship of seroprevalence and age group was studied using general linear modeling. Student's *t*-test was used to determine the significance of the differences of the mean values on age groups.

RESULTS

All 507 forestry workers were men living and working in rural areas. The mean age was 44.8 years (ranging from 21-65). The average period of work was 14.4 years (ranging from 1 to 40) and 50% had worked from 8 to 20 years. Among them, 68% reported daily contact with domestic animals (cats, dogs, chickens, etc); 72.8% reported outdoor activities; 24.2% reported at least one tick bite, and 22% had drunk raw milk. No clinical cases related to the investigated infections were remembered.

In this sample, 2.4% of the sera were positive when tested with *R conorii* antigen, 3.4% with *R slovaca*, 3.0% with *R mongolotimonae*, 2.8% with *R massiliae*, 1.0% with *R helvetica*, 24.9% with *C burnetii*, 17.4% with *B burgdorferi,* and 2.6% with *F tularensis* (**Figures 2 and 3**). No sera reacted with the HGE antigen. The results of

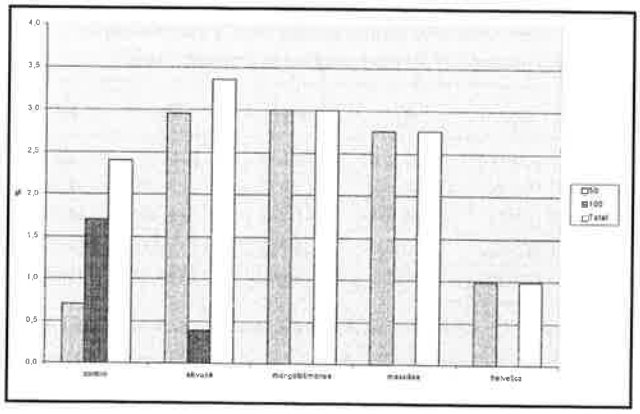

Figure 2. Seroprevalence of antibodies against spotted fever rickettsiae and distribution of serological titers in forestry workers in Tuscany, Italy.

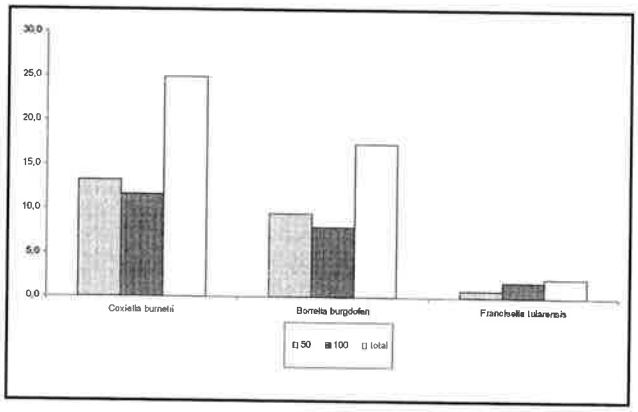

Figure 3. Seroprevalence of antibodies against Coxiella burnetii, Borrelia burgdorferi, Francisella tularensis, *and distribution of serological titers in forestry workers in Tuscany, Italy.*

Table 1. Seroprevalence of antibodies against spotted fever rickettsiae in forestry workers in different districts of Tuscany, Italy.

	Total	*R conori*		*R slovaca*		*R mongolotimonae*		*R massiliae*		*R helvetica*	
	n°	*n°*	*%*	*n°*	*%*	*n°*	*%*	*n°*	*%*	*n°*	*%*
Arcidosso	108	2	1.9	4	3.7	2	1.9	3	2.8	0	0.0
San Marcello P.se	67	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Barga	57	3	5.3	3	5.3	4	7.0	1	1.8	0	0.0
Abbadia S. Salvatore	30	0	0.0	0	0.0	0	0.0	0	0.0	1	3.3
Villafranca L.	40	2	5.0	2	5.0	2	5.0	1	2.5	1	2.5
Fivizzano	94	4	4.3	3	3.2	6	6.4	9	9.6	3	3.2
Acquerino	29	1	3.4	5	17.2	1	3.4	0	0.0	0	0.0
San Rossore	30	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Alberese	18	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Abetone	34	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total	507	12	2.4	17	3.4	15	3.0	14	2.8	5	1.0

the study in the different areas are reported in **Tables 1 and 2.** Cross-reactions were reported among spotted fever group rickettsiae, particularly between *R conorii, R slovaca,* and *R mongolotimonae* ($P < 0.05$, each other), but also between *R mongolotimonae* and all other rickettsiae and between *R massiliae* and *R helvetica* **(Table 3)**. A significant correlation was also found between seropositivity for *R massiliae* and *B burgdorferi* ($P < 0.05$). Considering the total of patients seropositive for *R conorii, R slovaca,* or *R mongolotimonae,* there was a correlation with seropositivity for *C burnetii* **(Table 4)**.

Seropositivity for *Coxiella burnetii* was correlated with age ($r = 0.89$). The seroprevalence was higher in patients older than 40 years (41.8% versus 20%; $P < 0.001$). Outdoor activities and contact with animals appeared as significant risk factors among the total of patients seropositive for *R conorii, R slovaca* or *R mongolotimonae* ($P < 0.05$). Outdoor activities were also significantly correlated with seropositivity for *Francisella tularensis* ($P < 0.05$) **(Table 5)**. No other significant correlation was found between the seropositivity for spotted fever group rickettsiae, Q fever, Lyme disease, tularemia, and the variables considered **(Table 5)**.

DISCUSSION

This study supports the presence of tick-borne bacterial diseases investigated among forestry workers in Tuscany, except human granulocytic ehrlichiosis. Forestry workers are a population at high risk for a wide variety of occupational related illnesses and particularly for tick-borne zoonoses. The area of investigation consists of a typical Mediterranean area, with a characteristic climate of cool and moist winters and dry and hot summers. Climatic factors and typical vegetation provide an ideal habitat for ticks and animal hosts. In Tuscany, hilly areas are predominant (67%) whereas plains and mountains are respectively 8% and 25%. In coastal and hilly areas shrub vegetation ("macchia mediterranea") is predominant with

Table 2. Seroprevalence of antibodies against the HGE agent, **Borrelia burgdorferi, Coxiella burnetii,** *and* **Francisella tularensis** *in forestry workers in Tuscany, Italy.*

District	Total	*Coxiella burnetii*		*B burgdorferi*		*F tularensis*	
	n°	*n°*	*%*	*n°*	*%*	*n°*	*%*
Arcidosso	108	36	33.3	18	16.7	3	2.8
San Marcello P.se	67	10	14.9	14	20.9	2	3.0
Barga	57	15	26.3	6	10.5	2	3.5
Abbadia S. Salvatore	30	9	30.0	5	16.7	0	0.0
Villafranca L.	40	14	35.0	5	12.5	1	2.5
Fivizzano	94	19	20.2	23	24.5	5	5.3
Acquerino	29	7	24.1	3	10.3	0	0.0
San Rossore	30	7	23.3	3	10.0	0	0.0
Alberese	18	5	27.8	1	5.6	0	0.0
Abetone	34	4	11.8	10	29.4	0	0.0
Total	507	126	24.9	88	17.4	13	2.6

oak and pine woods. In mountains below 1000 m of altitude, oak and chestnut woods dominate. Beech woods are predominant in areas over 1000 m a.s.l. Conifers are present over 1700 m a.s.l. There is a high population density of wild ungulates: fallow deer, roe deer and wild boar, muflon, red deer and small mammals: squirrels, mice, rats, martens, porcupines, hedgehogs. Domestic animals such as sheep and cattle are present in small breeding farms but more consistent data about their distribution are lacking. *Ixodes ricinus* is the dominant tick species in this area. Other ticks of medical importance such as *Rhiphicephalus sanguineus, Dermacentor marginatus,* and *Dermacentor reticulatus* are also present.[22,23]

The ecological characteristics of this region are substantially similar to those of other Mediterranean countries particularly southern France where emerging rickettsioses have been described in the recent years. Mediterranean spotted fever caused by *R conorii* is well known in Italy and more than 1000 cases of Mediterranean spotted fever are reported each year since 1989.[24] The disease is endemic in almost all the Italian regions and the higher prevalence is in the south of Italy, mainly in Sardinia and in Sicily (18.3% in healthy donors).[25] In other European Mediterranean countries seroprevalence varies from 4.2% to 45.3% depending on the region and cut-off used.[1,26-30] In this study, seroprevalence for *R conorii* in forestry workers was 2.4%. However, from 1992 to 1997 the clinical reported cases of Mediterranean spotted fever in Tuscany were less than 10 per year.[31] Rickettsiae cross-react with each other particularly when strains are antigenically closely related such as *R conorii, R slovaca,* and *R mongolotimonae*, and serological tests do not distinguish the different rickettsiae.[21]

Table 3. Cross-reactivity among spotted fever group rickettsiae antibodies among 507 forestry workers in Tuscany, Italy.

	Rc	*Rs*	*Rmo*	*Rm*	*Rh*
Rc	12 (100%)	7 (41.1%)	5 (33.3%)	1 (7.1%)	1 (20%)
Rs	7 (58.3%)	17 (100%)	5 (33.3%)	1 (7.1%)	1 (20%)
Rmo	6 (50%)	6 (35.3%)	15 (100%)	7 (50%)	2 (40%)
Rm	1 (8.3%)	1 (5.9%)	6 (40%)	14 (100%)	2 (40%)
Rh	1 (8.3%)	1 (5.9%)	1 (6.6%)	2 (14.3%)	5 (100%)

Rc: R conorii; *Rs:* R slovaca; *Rmo:* R mongolotimonae; *Rm:* R massiliae, *Rh:* R helvetica.

This is a preliminary observation that needs further examination. Ticks are known to be vectors but also reservoirs of spotted fever group rickettsiae (through transstadial and transovarial transmission) and ticks species that are known to harbor and transmit rickettsiae (*R conorii, R slovaca, R helvetica*) are prevalent in Tuscany. Screening ticks for the presence of the bacteria will support the presence of the diseases. Moreover, clinicians have to be aware of atypical tick-borne spotted fever and unspecific febrile illness and all available tools to identify emerging rickettsiae, particularly molecular methods.[1]

At the end of World War II in Italy, outbreaks of Q fever involved the Italian population as well as allied troops. The epidemic period lasted until the early 1960s. Since 1960 only sporadic clinical cases have occurred, suggesting an endemic situation even though an outbreak in northern Italy was recently described.[32,33] However, there are few sporadic cases reported despite the recent outbreak in the northern Italy.[34] Our study describes a high seroprevalence (24.9%) of the *C burnetii* infection in forestry workers in Tuscany. These data confirm a previous study conducted in Tuscany on people professionally in contact with ovines (shepherds, veterinarians, farmers).[33] Seropositivity for antibodies against *C burnetii* was higher in this risk group (49.1%) than in urban residents (6.1%); the serological test used was microimmunofluorescence and the cut-off titer was 1:20. In this study, we used the reference method for serological diagnosis of Q fever, immunofluorescence with a titer of 1:50 for cut-off.[18,35-39] The seroprevalence of 24.9% confirms the high incidence of this infection among these workers. Although consumption of raw milk is a well-known risk factor for Q fever, it was not correlated in our study with seropositivity for *C burnetii*.

The first case of Lyme disease was described in Italy in 1983.[40] Thereafter, numerous cases have been reported, especially in northern Italian regions.[41] In the present serosurvey, we detected a prevalence of 17.4% (range 5.6%-29.4%). In a previous study among 809 forestry workers living in 13 different geographical areas of

Table 4. Cross-reactivity between spotted fever group rickettsiae **Borrelia burgdorferi, Coxiella burnetii,** *and* **Francisella tularensis** *antibodies in 507 forestry workers in Tuscany, Italy.*

	Cb %	*Bb %*	*Ft %*	*Rh %*	*Rm %*	*RcRsRmo %*
Cb	126 (100%)	12 (13.6%)	3 (23%)	1 (20%)	0	10 (58.8%)
Bb	12 (9.5%)	88 (100%)	2 (15.4%)	1 (20%)	7 (50%)	5 (29.4%)
Ft	3 (2.4%)	2 (2.3%)	13 (100%)	0	0	1 (5.8%)
Rh	1 (0.8%)	1 (1.1%)	0	5 (100%)	1 (7.1%)	1 (5.8%)
Rm	0	7 (7.9%)	0	1 (20%)	14 (100%)	2 (11.7%)
RcRsRmo	10 (0.8%)	5 (5.7%)	1 (7.7%)	1 (20%)	2 (14.2%)	17 (100%)

RcRsRmo: sera reacting with R conorii, R slovaca *and* R mongolotimonae*; Rm:* R massiliae, *Rh:* R helvetica*; Cb:* C burnetii*; Bb:* Borrelia burgdorferi*; Ft:* Francisella tularensis.

Tuscany, the seroprevalence for *B burgdorferi* antibodies was 18.3%.[42] Although the seroprevalence in our region is high, the clinical cases are relatively rare. Moreover, our study yielded an association of seropositivity for *Borrelia burgdorferi* with seropositivity for *Coxiella burnetii* and *Rickettsia massiliae.* This may be explained by exposure to the same risk factors or the presence in this area of another bacteria responsible for cross-reactions between these organisms. *Borrelia burgdorferi, Rickettsia helvetica* and the HGE agent have the same vector (*Ixodes ricinus*). However, in our survey no significant correlation was found between seropositivity for *Borrelia burgdorferi* and *Rickettsia helvetica* and no sera reacted with the *Ehrlichia* antigen. This result must therefore be interpreted with a certain caution, since cases of HGE have been described in Slovenia,[12] which is very close to Italy. Moreover, the HGE agent has been detected in ticks in Italy and *E phagocytophila* DNA has been detected by PCR in 25% of free-living nymphs in the Lazio Region on the southern border of Tuscany.[15,43-46] A previous serosurvey conducted in the regional park of the Belluno district attributed the presence of antibodies to *E phagocytophila* in 20.5% and 89% of forestry workers, in 4.3% of civil protection workers, 5.5% of hunters, and 1.5% of residents.[44-46] In the same area, in another study conducted in 1996-1997, none of the 1,088 sera reacted with HGE (Webster strain).[15,47] These discrepancies in seroepidemiological studies may be explained by the fact that some antigens (*E phagocytophila, E equi*) might be more sensitive for diagnosis and epidemiology in Europe than the American strain (HGE Webster strain).[15,46] Thirteen (2.6%) sera reacted against *F tularensis* suggesting a real though moderate circulation of this agent. Sporadic cases of the ulceroglandular form of tularemia are identified annually in Italy.[24] Similarly in Tuscany few sporadic cases are noticed each year even if some epidemic foci

Table 5. Risk factors among forestry workers in Tuscany tested for the presence of antibodies against **Borrelia burgdorferi (Bb), Coxiella burnetii (Cb),** *and* **Francisella tularensis (Ft), R helvetica (Rh), R massiliae (Rm),** *and* **R conorii, R slovaca,** *and* **R mongolotimonae (RcRsRmo).**

	Cb		*Bb*		*Ft*	
	Pos	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Serology	126	381	88	419	13	494
Milk	34 (27%)	78 (20.4%)	20 (22,7%)	92 (21.9%)	5 (38.4%)	107 (21.6%)
Ticks	34 (27%)	89 (20.5%)	15 (17%)	108 (25.7%)	2 (15.4%)	121 (24.5%)
Animals	93 (73.8%)	252 (66.1%)	57 (64.8%)	288 (67.8%)	11 (84.6%)	334 (67.6%)
Other risks	98 (77.7%)	271 (71,1%)	68 (77.3%)	301 (71.8%)	13 (100%)	356 (72%)

	Rh		*Rm*		*RcRsRmo*	
	Pos	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Serology	5	502	14	493	17	490
Milk	1 (20%)	111 (22.1%)	2 (14.3%)	110 (22.3%)	5 (29.4%)	107 (21.8%)
Ticks	2 (40%)	121 (24.1 %)	3 (21.4%)	120 (24,3%)	6 (35.5%)	117 (23.9%)
Animals	3 (60%)	342 (68.1%)	9(64.3%)	336 (68.1%)	17 (100%)	328 (66.9%)
Other risks	5 (100%)	364 (72.5%)	10 (71.4%)	359 (72.8%)	17 (100%)	351 (71.6%)

have been described in the past.[48,49]

None of the subjects examined reported having had previous specific clinical symptoms compatible with the diseases studied in this work. Our results are comparable with those reported in other studies for Lyme borreliosis,[50] Q fever,[18] rickettsioses,[1] and HGE.[51] These infections may be subclinical or paucisymptomatic illness; they are seldom diagnosed or considered because of difficulties in clinical diagnosis, and in part because of the frequent unavailability of specific laboratory diagnostic tests. Also, the percentage of forestry workers with a history of tick bites in our study is very low (24.2%); a tick bite is usually painless and may not be noticed, particularly with nymphs.

The results of our study support the contention that there is a risk for Q fever and tick-borne bacterial infections among forestry workers and among people visiting these areas. Careful medical attention is needed and tick-borne diseases should be included among differential diagnosis of flu-like syndromes. In this study, we have reported some preliminary data on the seroprevalence of SFG rickettsiosis and Q fever that demonstrate the circulation of these infections and open a future prospective of work. Further data are needed for a better understanding of the epidemiology of these diseases in Tuscany. More specific serological tests such as Western blot will allow us to better define the true prevalence of these infections. In addition, further research on seroprevalence of other groups at risk are necessary in these and other areas, as in control groups (general population, blood donors) and in animal reservoirs. Finally, every effort must be made to identify the clinical cases. A greater awareness of these diseases and the use of newer and more sophisticated diagnostic tools such as molecular tools could greatly improve our knowledge of these infections in Tuscany.

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Perceptions of Lyme Borreliosis

Kara Bennett, PhD

ABSTRACT

Researchers have found traditional medical education offers little training in evaluating the dynamics of symptom patterns, especially nonlinear and unusual symptomotology. This can influence the diagnosis and treatment of the patient when physicians are presented with complex/fluctuating symptoms.[1,4,5] An investigation was performed to determine any relationship between the diagnoses and treatment received by patients eventually diagnosed with Lyme borreliosis, and the strategies physicians used to evaluate complex and fluctuating symptom patterns.

In this study, 10 patient diaries were used documenting the symptoms presented to the physician, every diagnosis, treatment, and reasons for them. Clinical diagnostic strategies suggested from previous research for evaluating complex symptom patterns were examined for each physician/patient encounter: whether the physician followed the patient's symptoms and signs over a period of time to look for different patterns; whether they only used the presence or absence of signs of illness; and whether they integrated their findings into multisystem diagnoses.

The 10 patients consulted a total of 106 physicians, presenting each physician with complex/fluctuating symptoms. Patients' symptom patterns were followed by 16 physicians who integrated specialty area findings for the diagnosis of Lyme disease, and offered long-term antibiotic treatment. Of the physicians who only looked for the presence or absence of signs of illness, 2 diagnosed Lyme disease. They did not treat the patient longer than one month with antibiotics.

Only specialty-area diagnoses not including Lyme disease was made by 79 physicians; whereas, 7 physicians who only looked for the presence or absence of signs of illness considered the patient had psychogenic problems. None of the physicians who followed symptom patterns concluded there were no medical problems.

This study supports previous research in the dynamics of disease.[1,4,5] It was suggested future research in Lyme borreliosis include the diagnostic strategies physicians use for evaluating complex/fluctuating symptoms.

Key words: Lyme borreliosis, dynamical diseases, *Borrelia Burgdorferi*

INTRODUCTION

Controversy surrounds the diagnosis and treatment of Lyme borreliosis, an arthropod vector spirochetal infection affecting both humans and animals. Diagnosing Lyme disease is often difficult because laboratory tests do not definitively show the presence or absence of the causative microorganism *Borrelia burgdorferi*.[2]

Lyme disease can present with complex and fluctuating symptom patterns and may mimic better known diseases including psychiatric disorders because the bacterium can disseminate throughout the body. Early diagnosis and treatment are essential for preventing long-term complications and possible life-threatening illness.[3]

The focus of this preliminary investigation is whether physicians who were interested in evaluating complex and fluctuating symptom patterns, meaning the presentation of a number of symptoms at one time that may change in morphology and severity over a period of time, were more likely to include Lyme disease as a possible diagnosis.

The variety of symptom patterns a patient might present could be viewed on a continuum from a simple linear pattern to nonlinear and chaotic. Although practitioners may look for patterns in the changes of a patient's symptoms, some physicians may view unusual changes with skepticism or not indicating serious illness, since traditional medical education has not often included methods for evaluating nonlinear symptom patterns.[1,4,5]

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Physicians interested in nonlinear symptom patterns have introduced the concept of a dynamical disease to describe illnesses that have unusual abnormalities in the control mechanisms of physiological variables, observed in the temporal patterns of the patient's symptoms and signs. For example, multiple sclerosis, manic-depression, and epilepsy have periodic symptom patterns. The ability to diagnose and treat a dynamical disease requires information about the patient's symptoms over a period of time to capture variations in quality and severity.[1]

Imagine for a moment looking through a dynamical "lens" at the patient who presents with complex and fluctuating symptom patterns versus looking at the same patient from a linear perspective where only an increase or decrease in severity of the symptoms is evaluated. For example, a patient eventually diagnosed with Lyme disease described one of her symptoms as "The uncontrollable jerking movement is back. This time it's accompanied by twisting head motions. At first it's intermittent, and I try to keep working in spite of it." One doctor's assessment was "I've just never seen anything like this before. There's a chance a seizure disorder could cause something like this although I doubt it. I still think this is a psychosomatic problem."[6]

Another physician approached her symptoms in a different way. He asked whether the movement problems subsided at night and increased when she went out in public. He explained that this pattern was similar to some of his patients' with Parkinson's disease. This doctor eventually helped the patient find the diagnosis of Lyme disease, based on both her unusual symptoms as well as flu-like symptoms, fatigue, joint pains, a positive serology, and being in an area where there were ticks that carried the disease. In contrast to the first physician, he considered unusual symptom patterns important diagnostic criteria.

Although Lyme disease has not been studied as a dynamical illness, the organism causing Lyme disease has been described as having unusual qualities. Borreliosis was known for at least 100 years before the strain of *Borrelia burgdorferi* was identified in 1981, and was described as "a dynamic condition, a cyclic disease characterized by a cyclic causative agent."[7] More recently Casjens et al,[8] completed the identification of the genome of *Borrelia burgdorferi* and found that the organism carries the largest number of extrachromosomal DNA elements known for any bacterium.

Given the possible multisystemic involvement of this illness and the unusual qualities of the causative organism, it would be important to better understand the ways physicians responded to the dimension of complex/fluctuating symptomotology in the process of diagnosing the patient.

BACKGROUND RESEARCH

The ability of a physician to evaluate different types of symptom patterns as part of the clinical diagnostic process has not been studied as a specific type of problem-solving skill. For example, depending on whether the physician decided to evaluate the symptoms on only one occasion or over a period of time would help determine whether patterns could be observed. The variables of whether the patient's problem was seen as only a specialty area problem, or if specialty area findings were integrated into a more encompassing disease category, could help determine how many signs of illness were included in the diagnoses given to the patient.

Traditional research of medical diagnostic processes focused on matching lists of symptoms to the most likely disease categories, and used this model for creating computer expert systems.[9] However, the symptoms, signs, and disease categories were considered static, *eg*, signs of illness were noted as present or absent, and their dynamics were not considered important information. To offer better accuracy in the diagnostic model, fuzzy representations of symptoms were included, along with a beginning analysis of their dynamic changes.[10]

In the real-life setting, only matching signs of illness to the most likely disease categories have been criticized for not offering in-depth investigation of the individual's history, nor being able to capture the complexity of the patient's problem.[11,12]

To evaluate complex and fluctuating symptom patterns, patient diaries are often helpful for obtaining the necessary type of information because they provide a record of the patient's symptoms over a period of time.[4] Since there has not been formal training in the use of dynamics theory in clinical practice, information about changes in symptoms over a period of time is not routinely included in the patient's medical records.[1,4,5] Even when there are devices available for recording time series information, such as the Holter monitor, a nonlinear dynamical analysis is not often performed.[13]

Some physicians have suggested to their patients ways of keeping a time series record. Sacks[5] provides an example of a patient who recorded the movement problems he experienced by noting the severity of the symptoms at 90-minute intervals over a three-month period. A phase portrait was created to show the pattern of changes that could then be compared to patterns of normal movement.

Patients eventually diagnosed with Lyme disease often kept diaries of their symptoms and diagnoses during the course of their illness. In many cases, their search took several years and some have published their health care stories to bring attention to the real-life experience of this illness.[6,14] Physicians have also written personal accounts

of their difficulties in being diagnosed with Lyme disease.[15]

These patient stories offer suggestions for finding how physicians evaluated complex/fluctuating symptom patterns, and whether their strategies influenced the choice of diagnoses. They provide important information not otherwise available and not routinely kept in medical records: observations of the daily-life experience of the illness, and statements made by the physicians regarding reasons for the diagnosis that include not only the kind of diagnosis, but also how the physician responded to the presentation of complex/fluctuating symptoms. Some patients were asked to keep records of their symptoms over a period of time. Others were told their myriad of symptoms were caused by stress and the patient was not evaluated further for medical problems.[6,15]

METHODS

The diaries of 10 patients diagnosed with Lyme Borreliosis were used for this investigation. The diaries were obtained by placing a request in a Lyme disease patient newsletter for people who had been diagnosed with Lyme borreliosis, based on clinical criteria and positive serology.[14] They were asked to send their medical history over the period of time taken to be diagnosed and treated for Lyme disease that included: symptoms presented to the physician, diagnoses they received, treatment given to the patient, and any comments made by the physician noted by the patient or included in their medical records regarding the reasons for the diagnoses.

To determine how the physicians evaluated complex and fluctuating symptom patterns, the diaries were examined for each physician/patient encounter to find: 1) Whether the patient presented complex and fluctuating symptom patterns; 2) How the physician evaluated complex and fluctuating symptom patterns; and 3) Information was combined to describe possible strategies used by the physicians to evaluate complex/fluctuating symptoms in finding the diagnoses of the patient's problems.

(1) Whether the patient presented complex and fluctuating symptom patterns.

Symptom presentation was defined as complex if the patient was concerned with three or more symptoms at the same time, such as nerve-like pain, vision problems, flu-like feelings, difficulty walking, fatigue. There are many definitions of complexity, including mathematical definitions.[16] Since this study focuses on the clinical problem-solving strategies of the physician, the common dictionary definition was used.[17] Fluctuating symptoms were defined as differing degrees of severity for the same symptom over a period of time

(2) How the physician evaluated complex and fluctuating symptom patterns.

The response to the patient's symptom presentation was examined for two types of clinical diagnostic strategies suggested by previous research for evaluating fluctuating symptom patterns[5,10,12] including (a) whether the physician followed the patient's symptoms and signs over a period of time to obtain the information necessary to determine possible patterns, and (b) whether the physician looked for only the presence or absence of a sign of illness and did not evaluate different types of patterns for symptoms or signs.

To determine which strategy was used, the statements made by the physician to the patient and/or listed in their medical records about the reasons for the diagnosis that might indicate how they evaluated fluctuating symptoms were examined, along with the patient's statements as to whether the physician followed their symptoms over a period of time.

In a previous published case,[15] a statement from the physician such as, "He said he would not treat nonsymptomatic deer tick bites," indicates an approach of using the presence or absence of symptoms, without following the patient over a period of time. Whether the patient was asked to see the physician again, or have the results of the examination sent to another physician would indicate if the physician could have collected the type of data needed for observing patterns in the symptoms.

To find how complex symptom patterns were integrated into the disease category each diagnosis given to the patient was placed in one of three categories representing the number of medical findings included in the diagnosis:

1. No medical findings and considered psychogenic;
2. Specialty area diagnoses only, including specific psychiatric diagnoses, but not including Lyme disease;
3. Integration of the specialty diagnoses into disease categories or syndromes, including Lyme borreliosis.

Statements that indicate reasons for the diagnosis after evaluating the complexity of the patient's problem were examined. For example, some patient's were told their symptoms were very unusual, and some physicians included these findings in the diagnosis, while others dismissed them as psychogenic.[6,15]

(3) Information from 1 and 2 was combined to describe possible strategies used by the physicians to evaluate complex/fluctuating symptoms in finding the diagnoses of the patient's problems.

The strategies were viewed in two main choices of diagnostic paths depending on whether symptom patterns were explored over a period of time, or whether symptoms were viewed as only indicating presence or absence of

signs of illness. These choices were then related to the three categories representing the number of medical findings included in the patient's diagnoses.

RESULTS

The 10 people whose diaries were used in this investigation included 3 men and 7 women ranging in age from 24 to 66 who did not know each other. They were from 8 different states: 2 from New York and Pennsylvania, 1 from Wisconsin, Missouri, New Hampshire, Kansas, California, and Canada. They all reported having been in good health prior to their illness, and visiting areas where they could have been exposed to arthropods carrying the microbe causing Lyme disease. Four reported that other members of their family also had been diagnosed with Lyme disease. The diaries all contained the information requested, and also offered details of the individual's experience.

They usually chose a family practice physician or internist for their first visit. Each of the 10 patients consulted from 2 to 30 physicians before receiving the diagnosis of Lyme disease, totaling 106 physicians including specialty area physicians. All patients eventually tested positive for serology. After receiving the diagnosis of Lyme disease and treatment with antibiotics, all patients reported improvement. However, repeated courses of antibiotics were necessary for all 10 patients, and stopping the antibiotics resulted in a return of symptoms. The amount of time taken to find this diagnosis ranged from three months to nine and one half years, and the average cost before finding the diagnosis was $20,000.

Presentation of Symptoms

From the initial visit to a physician until being diagnosed as having Lyme disease, all 10 patients presented with complex/fluctuating symptoms. Each patient presented with at least three symptoms and described at least one as having varying degrees of severity (see **Figure 1**). All patients initially described their symptoms as flu-like or feeling exhausted along with a variety of more specific complaints. No psychological problems were reported as a separate concern from the illness, *eg,* some patients reported feeling anxiety along with feeling ill. Only two reported having a bull's-eye rash and insect bite.

Over the period of time taken to find a diagnosis and effective treatment, new symptoms appeared within days, months or years later. For example, movement problems in different parts of the body, nerve and joint pain, muscle twitches, loss of stamina, mental confusion, memory problems. The symptoms could appear suddenly, such as with nerve-like pain and fluctuate over a period of time. Sometimes the patient felt most symptoms had returned to some degree; at other times there was temporary relief and the person might feel they were recovering.

Evaluation of Symptoms

Case Study

This case study offers a more detailed example of the results. Of the 10 cases, it represents a best-case scenario of a patient being diagnosed and treated for Lyme borreliosis. This patient initially presented with a severe sore throat, flu-like symptoms, and painful aching hips. She had a vague memory of a rash the month before but was not sure. Ticks carrying the *Borrelia* spirochete were documented as being found in the area, including one found on their land. She did not initially test positive for Lyme disease, then later was positive with IFA of 512.

Throughout her illness this patient had one family practitioner who continually monitored her progress and treatment. The patient describes this physician as one who would listen to her concerns seriously and explore a variety of possibilities. Although he did not initially suggest Lyme disease as a diagnosis, he arranged for tests after it was suggested by a veterinarian within the first few months of her illness. He also referred her to different specialists to assess specific problems and help confirm the Lyme diagnosis.

The specialist's response to her symptoms included: 1) a rheumatologist decided her problems were caused by stress because she did not have a high sedimentation rate at that time, and therefore, stated she couldn't have an infection; 2) a cardiologist found heart abnormalities and decided they were not related to Lyme disease because they didn't fit what he had read about Lyme disease; 3) a gastrointestinal physician thought she might have Crohn's disease instead, then after ruling it out, believed the case "fell outside known parameters"; 4) an endocrinologist ruled out hormonal changes; 5) a neurosurgeon found and removed a benign lump from her neck that he considered unusual and stated he had not seen anything like it before; 6) a neurologist and internist looked for patterns in her symptoms such as difficulty walking, weakness, and joint pain, and agreed her symptoms could be consistent with Lyme disease.

Her family physician included the specialists' findings in an overall evaluation of her illness, and continued to follow the patient's reports of fluctuations in her symptoms. He believed Lyme disease was one of the best fits to a diagnostic category. He was curious to keep learning about Lyme disease while continuing to evaluate other possible causes. The patient needed repeated courses of antibiotics for any sustained improvement, finding thatstopping the antibiotics resulted in an exacerbation of symptoms.

Figure 1. Symptoms presented over the period of time leading to diagnosis and treatment of Lyme disease.

(Listed in chronological order of presentation)

Case #1—Time to diagnosis: 3 months
Fatigue, sleeping 14-15 hours per day
Mental confusion
Arthritic hands
Severe lower back pain
Stiff neck
Bones crack
Sore throat
Headaches
Increased confusion

Case #2—Time to diagnosis: 12 months
Numbness in arms while sleeping
Heart palpitations
Terrible dizzy spells
Strange sensations in head
Overall feeling terrible
Eyes felt weird
Problem with double vision
Almost a glow around objects
Intestinal problems

Case #3—Time to diagnosis: 22 months
Bull's eye rash on cheek, covering side of face
Fleeting symptoms for a year
Severe back pain by sacral joint and hips
Hurt everywhere
Tender around rectum and coccyx
Symptoms increased around time of menses

Case #4—Time to diagnosis: 17 months
Flu-like symptoms
Headaches, fever, cough, joint, and muscle pain
Leg cramps
Vision problems

Case #5—Time to diagnosis: more than 2 years
Severe crippling joint pains (intermittent)
Serious neurological impairment
(memory loss and sleep disorders)

Case #6—Time to diagnosis: 2 years
Fevers, severe morning stiffness, breast infection
Hand pain
Headaches
Neck pain
Eye pain
Right side head pain
Total body pain
Ankle swelling
Legs weighted (could barely walk)
Stiff
Pain like bone cancer, swelling, blood vessel bruising
Neuropathies, weakness
Severe nighttime ataxia
Irregular heart beat
Inflammatory responses on ligaments
Tendons giving out
Morning stiffness
Joint swelling in evenings
Hip tightness
Headaches
Neck pain continued
Foot infection
Rib spreading pain
Swelling of bone and ligament along lumbar spine
Mental confusion
Neuro weakness of entire body
Shuffling gait
Hot flashes
Weak leg, stiff neck, headache
Confusion, disorientation
From lying position, reel around, cannot keep balance
Urinate about 27 times per day
Bleeding when having trouble starting to urinate

Case #7—Time to diagnosis: 15 months
Flu-like symptoms
Left chest and arm pains
Tightness and shortness of breath
Twitches
Loss of concentration
Extreme fatigue
Diarrhea-like stool
Numbness in both arms and hands
Rare strange feeling in lower left cheek
Symptoms returned after 6 months of feeling 80% okay
Sinus infection (self-diagnosed)
Lower body aches (during IV Rocephin)
Felt "pins" in left thigh
Run down after 3 weeks of feeling good
Concentration problems
Previously injured areas on left hand, right wrist, and low back ache much of the time
Hot sensation in cheeks

Case #8—Time to diagnosis: 26 months
Vomiting
Dizzy
Ache all over
Violent headache
Weakness
Confusion
Anger
Flu-like symptoms
Headache, vomiting, weakness, much confusion

Figure continues

Figure 1 (continued). Symptoms presented over the period of time leading to diagnosis and treatment of Lyme disease.

Days spent in bed
Pain in head and eye
Great pain on whole left side of face
Face so sensitive could not tolerate daylight, air blowing, touching
Could not eat
Blood pressure up and down
Problems with light
Frustrated and angry
Chest pains
Hard to breathe
Confusion and fatigue
Disrupted sleeping pattern
Cold
Heart pounding
No control on left side of face
Right fingers numb
Great eye pain

Case #9—Time to diagnosis: 5 months
Raging sore throat
Flu-like symptoms, especially painful aching hips
Vague memory of rash one month earlier
Sporadic aching of joints, especially at night
Right knee hurt
Tenderness in front of arm pits progressed to general aching of shoulders and upper arms
Hip ached more, muscles ached
Shooting pains down arms into hands and fingers
Fatigue, pain intense
Intolerable pain and fatigue
Lump in neck
Depression—cyclic
Intense abdominal pain
Rectal bleeding, persistent severe abdominal pain

Case #10—Time to diagnosis: 9 years 7 months
Spreading red rash
Fever
Back strain
Chest pains with sore throat, fever, and vomiting
Mysterious rash over entire body
Severe stomach pains, heavy bleeding with clotting
Pain radiates down into hips
Chest pains and pain in shoulders
Skin legions in vaginal area (four separate times)
Skin lesions over arms, shoulders, and face
Swelling in knees and hip
Numbness in hands and legs
Hives and welts on back and buttocks
Rash on both eyes, peeling, and itching
Severe headache with vomiting
Violent shaking on right side of body
Bowel problem, blood in stool, diarrhea
Flu-like symptoms, chest pains, and fever
Rashes (bright red)
Numbness and tingling feeling in legs
Double vision and bubbling of lens of eye
Short-term memory loss, joint pains, and lower back pain
Stiff neck, severe headache, and vomiting
Hip and back pain
Shooting pains in head
Sore throat, cough, congestion, and fever
Pain in lower back and down right leg
Excess hair growth on face and chest
Vaginal dryness
Cracking joints/muscle cramping, burning, twitching, numbness, and tingling in hands, arms, and legs
Buzzing and ringing in ear
Memory loss, fatigue, and nightmares

The patient in this case study had one primary physician who followed the changes in her health throughout her illness, seeking further information when he did not know what was wrong, and integrating the findings of specialists into an overall diagnosis that included a cause for most of the patient's problems. Of the 7 specialists, 2 looked for changes in the symptoms over a period of time and helped fit the findings to the Lyme disease category. The other 5 did not look for patterns over a period of time, but whether medical findings were either present or absent for any one examination; 2 stated they had not seen the findings before, but did not try to look for more information; 1 decided the patient's problems were caused by stress; 1 concluded that the abnormal findings were not related to Lyme but did not suggest another disease category; and 1 helped rule out hormonal changes.

Summary of Ten Cases

No Medical Findings—Considered Psychogenic. Seven patients were told during their search for a diagnosis their problems were psychogenic, such as stress and unspecified mental problems. The patients did not report feeling less ill during these visits to a physician.

This type of diagnosis was made by family practice physicians, internists, rheumatologists, and neurologists. The physicians stopped any further evaluation after a routine examination with no medical findings they believed significant. They did not look for patterns in the patient's symptoms, but looked for either the presence or absence of a specific sign of illness they considered important, such as having a high sedimentation rate to indicate infection. They also did not integrate any findings into a disease category. From comments made to the patients or

listed in their medical records, four reasons were given for this type of diagnosis (numbers in brackets indicate number of physicians):

Too many symptoms ("No one could have all those symptoms.") [1]

Unusual symptoms ("I've never seen this before.") Not as a statement of interest but implying it was not a real problem. [1]

No reason to pursue further diagnostic tests because the person has too stressful a life ("You're working too hard".) [3]

The patient is doing this to herself ("You're enjoying the role of the invalid.") ("The patient thinks she's in pain.") [2]

Only Specialty Area Diagnoses *(not including physicians who helped diagnose or confirm Lyme disease)*

All of the patients were diagnosed with a variety of specialty area problems that included neurology, psychiatry, rheumatology, infectious disease, endocrinology, ENT, gynecology, cardiology, dermatology. No one specialty area diagnosis was the same for all patients. The most common diagnosis was fibromyalgia for four patients. Each had unusual findings considered a medical problem rather than psychogenic.

Five physicians followed the patient's symptoms over a period of time using tests such as the Holter monitor, repeating laboratory tests, and asking the patient to keep records of the fluctuations in their symptoms. There was no suggestion of any formal dynamical analysis nor reasons why this approach was taken, but rather an interest was shown in tracking unusual patterns and spending time listening to the patient. However, these physicians did not include their findings in a more encompassing disease category.

Although 74 physicians did not follow their patients over a period of time, they used the presence or absence of a sign of illness as their diagnostic criteria. Their reasons were:

Medical findings are consistent with the criteria for a specific specialty diagnosis, *eg,* heart problems [43].

No medical findings, which helps rule out an illness. [21].

Findings are unusual and do not fit a known category, but are believed to be a medical problem [10].

Diagnosis of Lyme Borreliosis and Other Multisystemic Illness

The diagnosis of Lyme disease was made by 18 physicians, including specialists. Patients had at least one positive serology with IFA, ELISA, or Western Blot tests. Two had positive urine antigen tests. Two patients had what they described as a bull's-eye-type rash. One was initially told it was not ringworm or a spider bite, and the physician was not sure what it was. The other patient was told the rash was a spider bite.

In two patients, the physician used the presence or absence of a positive serology test as the primary diagnostic criteria and prescribed a specified length of treatment with antibiotics. When this treatment length that ranged from two weeks to one month did not resolve the symptoms, or symptoms returned after stopping the antibiotics, the patients were told they no longer had Lyme disease, and must have other problems. This resulted in their seeking other physicians for longer-term antibiotic treatment.

The 16 physicians who diagnosed and treated Lyme disease with repeated courses of antibiotics followed the fluctuations in the symptoms over a period of time, including repeated testing of the Lyme disease. If they were also specialists they helped integrate the signs of illness into the overall diagnosis. Their reasons for the diagnosis included the overall clinical presentation of the patient's signs of illness, along with laboratory findings, and history of being in an area where they could have been exposed to the illness. These physicians did not suggest any formal dynamics analysis, but expressed an interest in exploring the symptom patterns and unusual findings.

Five of the patients requested being evaluated for Lyme disease because (a) a friend had read about the illness and suggested it to them, (b) read about the illness and thought it sounded like their symptoms, and (c) others in the neighborhood had already been diagnosed for Lyme disease. The other five patients were diagnosed initially by an emergency room physician, a neurologist, family practice physician, ophthalmologist, and internist. For two patients, a multisystem illness was also diagnosed as chronic fatigue syndrome.

SUMMARY

All 10 patients presented with complex and fluctuating symptoms. Most physicians [83] used the approach of looking only for the presence or absence of a sign of illness that fit their diagnostic criteria, and did not integrate the medical findings into a more encompassing disease category. There were 74 physicians who found only specialty diagnoses or helped rule out a possible diagnosis, 7 who believed the patient had only unspecified psychological problems, and 2 who diagnosed Lyme disease based on positive serology.

Patient's symptoms over a period of time were followed by 23 physicians who looked for different symptom patterns: 5 gave them only a specialty area diagnosis and did not integrate the medical findings, 2 suggested a multisystemic illness of chronic fatigue syndrome, and 16 diagnosed Lyme borreliosis. The physicians who followed the patient's symptom patterns also adjusted treatment of

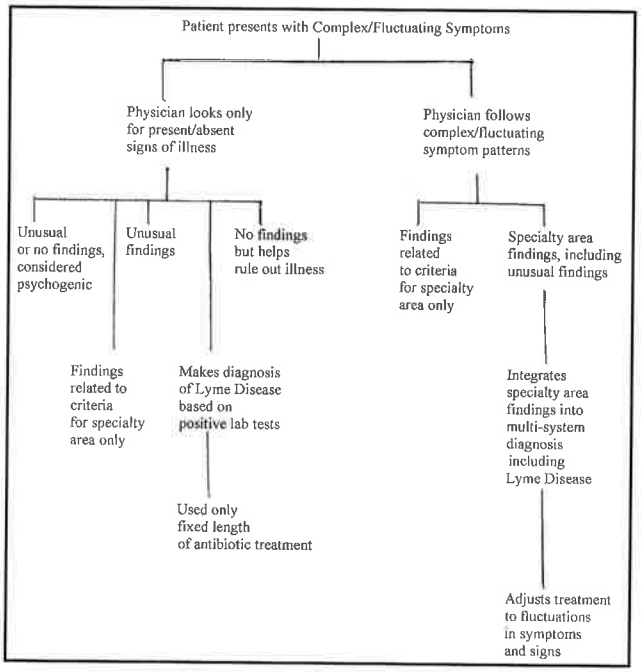

Figure 2. Clinical diagnostic and treatment strategies.

antibiotics to the changes in the patients symptoms, and used long-term treatment; whereas, the two physicians who only used the presence of absence of a sign of illness without following symptom patterns, used only short-term antibiotic treatment (see **Figure 2** and **Table**).

DISCUSSION

The results of this study show a possible relationship between the type of strategies used by physicians to evaluate complex/fluctuating symptom patterns, and the diagnosis and treatment of the patient.

The choice to explore different types of symptom patterns was made by only 23 of the 106 physicians consulted by the 10 patients. This finding fits previous statements made by physicians interested in the nonlinear dynamics of disease, that medical education has not often trained physicians to analyze different kinds of symptom patterns.[1,4,5] There was no formal application of dynamics theory, but physicians interested in symptom patterns were also more interested in the patient's record of symptom changes, and in pursuing more information about unusual findings.

Sixteen of these physicians also integrated the specialty findings and diagnosed Lyme disease. For example, if the patient had difficulty walking part of the time, they did not dismiss the changes in severity, but used this as a pattern of change, rather than needing to find only if the patient could walk or not walk on any one occasion.

Table. Number of physicians using each diagnostic strategy.

Evaluation Choices	Psycho-genic	Only Specialty Area	Multi-system	Total
Follows symptom patterns	0	5	18	23
Uses only +/- signs of illness	7	74	2	83
Total	7	79	20	$n = 106$

The other two physicians who diagnosed Lyme disease based their diagnosis on a positive serology, and not on a clinical analysis of symptom patterns. The different criteria for diagnosing this illness was carried into the way of thinking about the treatment of the patient. The choice to follow symptom patterns over a period of time reflected the way treatment with antibiotics was adjusted to fluctuations in the patient's symptom pattern and the use of longer term antibiotic treatment. Whereas those physicians who only looked for a presence or absence of a particular sign, such as positive serology, defined the treatment as requiring only a fixed time limit. After that time, they believed the patient no longer had Lyme disease.

There are still no definitive laboratory tests for determining the presence or absence of the microbe causing Lyme disease,[18] and the ability to make a clinical diagnosis of Lyme disease remains essential.[19] Part of that ability may involve the methods used to evaluate complex and fluctuating symptoms. To discuss these methods in more detail, they will be described as how they helped the physician arrive at a particular category of diagnoses.

No medical findings considered psychogenic. All of the physicians who decided the patient had "mental" problems or stress, chose not to follow the patient after one office visit, and used only the presence or absence of a sign of illness to make this decision. Although Lyme disease can present with psychiatric problems, the diagnoses would usually be more specific, such as depression, and not the more general categories such as stress or psychogenic. It is possible that if a physician uses only the exact value of a sign of illness, and is then confronted with a myriad of symptoms that are not easy to measure, they might need to find a way to bring the patient's pattern of symptoms more in line with their diagnostic strategy and dismiss the symptoms as not significant.

The statements made by the physicians in this group indicate a rejection of the seriousness of the illness, such as "no one could have all those symptoms." This suggests a self-imposed boundary for the physician as to how many symptoms can be present at one time and be considered a medical problem. This is in contrast to those physicians who did not find signs of illness on their initial

examination and/or laboratory tests, but continued to pursue evaluation of the patient over a period of time.

Only specialty area diagnoses (not including physicians who helped diagnose or confirm Lyme disease). Most physicians diagnosed the patient only in a specialty area, using the approach of looking for the presence or absence of signs of illness, and not following the symptoms or signs over a period of time to look for different types of symptom patterns. This resulted in the patients having several specialty diagnoses, but no diagnosis for an underlying cause or syndrome.

When the physician did follow patterns of symptoms and signs but did not integrate the findings into a more encompassing disease category, they also did not find an underlying cause for the patient's problems. Strategies for integrating the different medical findings would need to be used to determine if there was an underlying cause, or syndrome. Findings that used repeated measures over time would then be helpful for future evaluation because they offer information to fit to a diagnostic category that includes complex symptomotology.

Diagnosis of Lyme Borreliosis and Other Multisystem Illness

Being able to fit the patients complex/fluctuating symptom patterns together in a diagnosis that included an underlying cause and resulted in treatment that helped improve the patient's health was accomplished by physicians who were able to follow the patient's symptoms over a period of time, and integrate their findings into an encompassing disease category.

The two physicians who diagnosed the patient with chronic fatigue syndrome rather than Lyme disease did not use different diagnostic strategies, and this indicates the need for more understanding of the specific dynamics of Lyme disease, and/or inclusion of more than one disease process for their diagnoses. Most physicians who believed the patient had Lyme disease, also accepted they could have other problems, but believed Lyme disease could also offer a treatment that often resolved most of their symptoms.

The patients who suggested Lyme disease as a possible diagnosis to their physician had kept records of their symptoms over a period of time because they considered their illness very unusual and severe for them, and they often had difficulty finding effective treatment. They stated that their symptoms and fluctuations fit what they or their friends had read about in other cases of Lyme disease. Physicians interested in evaluating the possibility of Lyme included both those who decided to follow their illness over a period of time, and physicians who did not follow any patterns in the symptoms and signs but agreed to test them for Lyme disease. However, the physicians that relied on the patient having a positive test for the diagnosis also did not offer long-term antibiotic treatment, so these patients had to find other physicians if they wanted longer treatment.

In summary, these preliminary findings suggest that being able to find and integrate different types of patterns for the symptoms and signs of the patient's illness is a more effective strategy for diagnosing and treating Lyme disease, than ignoring the patterns or only focusing on the most severe signs of illness. If Lyme disease were studied as possibly being a dynamical disease, perhaps these patterns could also be related to the changes of the microorganism causing the illness.

Validity of the Data

The use of patient diaries for research is more accepted by researchers interested in dynamical diseases (20), because they provide information over a period of time about the same symptoms and signs. Unless records are kept for the same symptoms over a period of time, the dynamics may not be possible to assess. For example, when a symptom is not reported in the medical records as significant, it may not be considered important information, yet knowing the changes in severity will help understand patterns in the symptomotology. It is the fluctuations in severity that the patient is often most aware, and would need to be documented in some way for any dynamic analysis to be possible.

Therefore, rather than being considered anecdotal, the patient's reports of their symptoms is the only preliminary data available at this time for investigating complex and fluctuating symptom presentation. In the future, if more physicians become interested in nonlinear symptom patterns, this information could be included in the medical records, and might help to find new patterns of change in an illness. It is also the only data available for studying how the physician responded to the presentation of complex/fluctuating symptoms. Since this subject is a recent area of inquiry, the physician would not be expected to note whether s/he requested a nonlinear dynamics analysis, for example, to look at changes in the patient's cardiac problems.

The behavior of the physician in choosing to follow the patient over a period of time, and statements made to the patient directly or recorded in their medical records is the type of information that can be used for a preliminary investigation. The physician's statements made to the patients in this study were also similar to statements reported previously by both patients' and physicians' accounts of their difficulties in finding a diagnosis of Lyme disease,[6,15] *eg,* several patients in this study were told they had unusual symptoms not seen before, were under too much stress, or had too many symptoms.

In the future, the use of methods for studying physician's problem-solving strategies that use video recordings of the physician/patient encounter to obtain a protocol analysis of the diagnostic process, would be helpful in providing more directly observed data.[12,21]

The question of whether these patients actually had Lyme disease is also important in determining the validity of the findings. Until there are more agreed upon methods for diagnosing this illness, the assumption that these patients did have Lyme would not be more of an assumption than is made in studies based on self report or survey data. There was no reason to assume these patients did not accurately report their experiences because they offered to share their medical records.

The diagnosis of Lyme disease was considered over a period of time to be one of the best choices of a diagnosis, but not necessarily the only problem. For example, these patients were not tested at the time of this study for other tick-borne diseases such as Babesiosis or Ehrlichiosis.

CONCLUSIONS

The continuing controversy surrounding the diagnosis and treatment of Lyme disease may have one additional issue to consider; clinical diagnostic strategies used by physicians to evaluate complex/fluctuating symptom patterns are important in determining whether the physician is able to diagnose and treat Lyme disease as a multisystem illness over a period of time, or can only use a static or exact value of a sign of illness such as a laboratory test for the diagnosis and inflexible time of treatment.

This study found the difference in the perception of the seriousness and underlying cause of the patient's illness depended on whether the physician was interested in looking for and evaluating symptom patterns, including unusual and possibly nonlinear patterns, rather than looking only for the presence or absence of signs of illness.

This finding supports previous research and interest in dynamical diseases that found most physicians are not trained to evaluate different kinds of symptom dynamics.[1,4,5] Although Lyme disease has not been studied as a dynamical disease, this could be an important area for future research. Documenting different types of symptom patterns could help show changes specific to Lyme borreliosis that along with more definitive laboratory tests might also show correspondence to the cyclic changes of the microbe causing the illness.

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Cultivation of East African Relapsing Fever *Borrelia* and Review of Preceding Events

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ABSTRACT

Despite numerous attempts to cultivate *Borrelia* over the years, some species remain elusive. The interest generated by Lyme borreliosis has aided improvements in the cultivation of these spirochetes. These in turn have allowed cultivation of some of the most notable members of this genus, namely *Borrelia recurrentis*, the cause of louse-borne relapsing fever, and *Borrelia duttonii*, the cause of East African tick-borne relapsing fever. We review the history of cultural attempts and the events leading to the successful cultivation of these spirochetes.

A single isolate of *B recurrentis* was first successfully cultivated in 1993 from the blood of an Ethiopian patient. By 1995 a further 17 isolates were cultivated validating the technique.

Similar methods were used to try to grow *B duttonii*, which was also considered noncultivable. Following a visit to Tanzania in 1998, 5 isolates were grown. Cultivation of these strains will allow the detailed study of these relapsing fever species, enabling their biology, epidemiology, and pathogenicity to be revealed.

Key words: *Borrelia duttonii, Borrelia recurrentis,* tick-borne relapsing fever

During the first half of this century, relapsing fever was a disease of great world-wide importance with repeated major epidemics that affected 50 million of the population with a 10% to 40% mortality.[1] Following the second World War, further outbreaks affecting approximately 10 million with a 5% mortality are believed to have occurred.[2] Since 1967 the epidemic form of louse-borne relapsing fever has been largely confined to areas of extreme poverty in East Africa and the Peruvian Andes, with most cases arising in Ethiopia. A recent outbreak in neighboring Sudan is estimated to have affected 20,000 individuals of the Dinka tribe during 1998-9, with a mortality of 10% to 14%. No re-emergence of louse-borne relapsing fever has occurred despite the reappearance elsewhere in the world of other louse-borne diseases such as epidemic typhus in Bosnia and trench fever in vagrants. Furthermore, molecular analysis of lice collected from around the world including France, Peru, Russia, and the African countries of Burundi, Congo, and Zimbabwe, failed to produce evidence of louse-borne relapsing fever.[3] Tick-borne relapsing fevers tend to be more sporadic, but still causes major health problems in Africa where in areas such as Central Tanzania, it is one of the major causes of child mortality.[4] Although present in some European countries and America, tick-borne relapsing fever tends to be more of a rarity. It is often associated with camping in rural locations in close proximity to animal reservoirs of the spirochete and their associated *Ornithodoros tick* vectors.[2]

A spirochetal cause of relapsing fever was first demonstrated by Otto Obermeier during the 1867-8 outbreak in Berlin. He recorded spirochetes in the blood of patients with clinical relapsing fever; however, the inability to reproduce the disease in animal models, and indeed

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in himself, delayed the publication of these findings until 1873.[5] Soon after, in 1879, Moczutkowsky confirmed transmission of the disease by inoculating blood from cases of relapsing fever into healthy individuals. Later, Ross and Milne discovered the causative agent in the African variety of relapsing (tick) fever.[6] This finding was also made independently by Dutton and Todd who demonstrated relapsing fever in monkeys transmitted by infected *Ornithodoros moubata* ticks.[7] The role of the human body louse in the transmission of relapsing fever was reported by MacKie in 1907.[8]

Following the discovery of the epidemic relapsing fever spirochete by Obermeier, no attempts to cultivate the organism were made until 10 years later when Koch introduced his cultivable approach to the determination of the etiology of infectious agents. Indeed, in 1879, Koch mentioned the propagation of *Spirochaeta obermeieri* as it was then known, on artificial media in a letter to the systematic botanist, Cohn in 1879. Further results were never presented and no further mention was made in Koch's subsequent writings, suggesting that further cultivable attempts had failed.

The next few decades saw the succession of discoveries of further relapsing fever spirochetes, namely *S duttonii, S kochi,* and *S novyi* (all now members of the genus *Borrelia*). Successful attempts to cultivate these and *S obermeieri* were reported by Noguchi in 1912[9] using a medium of either ascitic or hydrocele fluid and rabbit kidney; however, attempts to reproduce this work by others were unsuccessful. Subsequently, egg-albumen media supplemented with ascitic fluid, glucose, and a buffering system was found to give better results with prolonged persistence of spirochetes and up to three subsequent subcultures.[10] It is possible that the increasing numbers of spirochetes reported in these early studies were in fact "pseudo-multiplication" as a result of sedimentation of these organisms.

Survival appeared to be best in plain defibrinated blood, with one report of spirochetes persisting for 40 days. It was noted that the disease stage of the patient affected the subsequent survival of spirochetes with early stages of infection producing best results, presumably as a result of the lack of antispirochetal antibody.[10] Samples collected late in infection failed to maintain the spirochetes for a day. Strains were however maintained by the transfer of blood from patients to monkeys, and in turn, into rats. Spirochetes could be seen in the blood of these rodents within 40 hours and persisted for up to three days.

A major advancement was made by the studies of Richard Kelly who developed a culture medium capable of supporting growth of American tick-borne relapsing fever spirochetes, *Borrelia hermsii, B parkeri,* and *B turicatae.*[11,12] Yields of up to 10^7 were obtained with a generation time of 18 hours when medium was inoculated with a large inoculum. However, he was unsuccessful in his attempts to cultivate *B recurrentis*. This finding was reaffirmed by Dodge[13] who attempted to isolate *B recurrentis* from Ethiopian patients with louse-borne relapsing fever using Kelly's medium and different modifications of trypticase soy yeast broth. Although initially obtaining growth, she was unable to propagate these isolates. Kelly made two further modifications resulting in Kelly's media B and C designed for the propagation of *B hispanica* and *B recurrentis* respectively.[12] Kelly reported poor growth of *B recurrentis* in his medium C with cultures reaching 10^6 organisms per mL and a generation time of 26 hours. Further adaptation of Kelly's medium resulted in the Barbour Stoenner Kelly (BSK II) medium now used for cultivation of *Borrelia burgdorferi* sensu lato.[14]

Both tick-borne *B duttonii* and louse-borne relapsing fever are considered to be diseases in which man is the only reservoir. Both spirochetes are fastidious and until recently were considered noncultivable. However, unlike *B recurrentis, B duttonii* will infect laboratory mice. Prior to this study, research on *B duttonii* has been restricted by the amount of material that can be collected from mice. An in vitro cell culture method was described using Sf1Ep cells, which could provide more cellular material, but has not been widely adopted.[15] Many have preferred to propagate this spirochete through mice.[16]

In vitro cultivation of *B duttonii* in BSKII was attempted as recently as 1997, but unfortunately failed.[17] We now review the cultivation of *B recurrentis* from patients with louse-borne relapsing fever in Addis Ababa, Ethiopia[18,19] and *B duttonii*[20] from patients with tick-borne relapsing fever in Mvumi, Central Tanzania.

MATERIALS AND METHODS

Ticks/Lice

Lice were collected from the clothing of patients with louse-borne relapsing fever. An example of louse-infested clothing can be seen in **Figures 1A and 1B.** These were pooled in batches of up to 10 lice before being cleansed and processed for culture as described below. *Ornithodoros* ticks were collected from traditional mud-built "Tembe" dwellings in villages in the Mvumi Hospital catchment area, Central Tanzania. Earth removed from the floor and lower walls was sieved to separate out the ticks that could then be collected. A typical example of *O moubata* ticks is seen in **Figure 2.**

Lice and ticks were surface cleansed by shaking them in 1 mL of 70% isopropanol to reduce nonborrelial contaminants. Lice and small ticks were homogenized in 1 mL of medium using sterile tissue grinders, and this homogenate used to inoculate culture medium either with

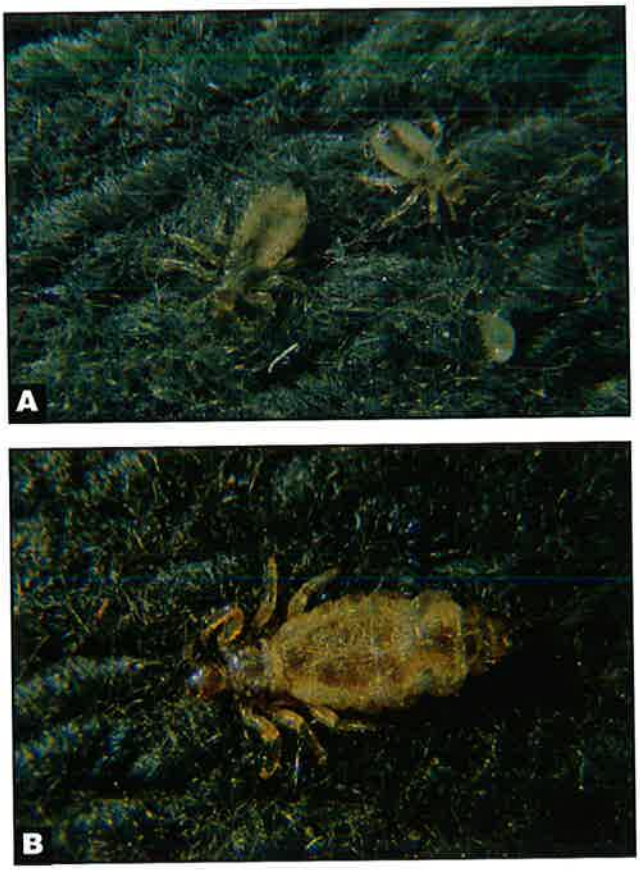

Figure 1. Louse-infested clothing from a patient with louse-borne relapsing fever in Ethiopia. (A) Shows nymphal stages and an egg while (B) shows an adult.

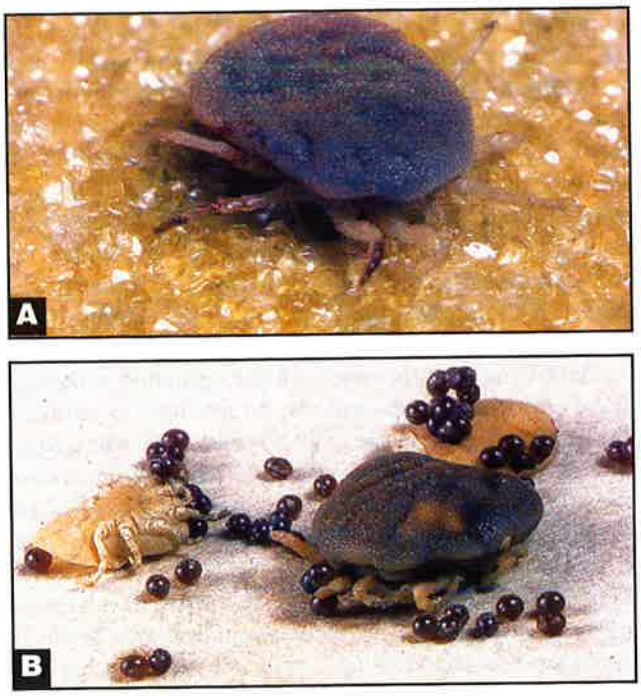

Figure 2. (A) O moubata *nymphal stage and (B) adult with eggs in plate. These were collected from traditional style dwellings in Tanzania, an area with endemic tick-borne relapsing fever.*

or without antibiotics to make it selective. Larger ticks were cut open, and the gut contents inoculated directly into culture medium as above. Cultural attempts from lice were inoculated into culture medium in Ethiopia and then transported to the United Kingdom while those from ticks were inoculated following the transport of ticks to the United Kingdom.

Patients

During visits to Addis Ababa in Ethiopia, patients presenting at accident and emergency, and those in hostels or local clinics suspected of having relapsing fever were finger pricked and blood films prepared. These were stained using Wright's or Field's stain, and if spirochetes were found, a blood sample was collected and the patients treated. Following centrifugation for 10 minutes at 1000-2000 rpm the buffy coat layer was inoculated into Kelly's medium[12] and into BSKII medium.[14]

Patients in Tanzania patients were examined in an outpatient clinic where a blood film was analyzed and 2-3 capillary blood tubes were filled if the individual was spirochetemic. Venepuncture was not attempted because of the young age and malnourished status of most patients. Whole blood was used to inoculate BSKII culture medium.

Culture Media

Kelly's media A and C, and BSK II media were prepared, filter sterilized and supplemented with autoclaved gelatin to give a final concentration of 1.7% (Kelly's) or 1% (BSKII), and sterile rabbit serum to give a final concentration of 6%. These were then aliquoted into polystyrene tubes each containing 6 mLs of media. **Tables 1-3** give the full composition of the media used.

Great variation has been noted in the ability of bovine serum albumin fraction V to promote growth of relapsing fever *Borrelia*. This has been found not only among products from different suppliers, but also among different lot numbers supplied under the same product code from the same supplier. As a consequence of these observations, each new batch or lot number of albumin was tested for its ability to grow these organisms before purchase for inclusion in BSKII medium.

Antibiotic supplements of rifampicin (to give a final concentration of 100 µg/mL), colistin sulphate (final concentration of 50 µg/mL), and 5-fluorouracil (final concentration of 100 µg/mL) were added to culture medium used

Table 1. BSKII medium.

Compound	Quantity per Liter
CMRL1066 without glutamine	100 mL
Neopeptone	5 g
Bovine serum albumin fraction V	50 g
Yeast extract	2 g
Hepes buffer	6 g
Glucose	5 g
Sodium citrate (trisodium salt)	0.7 g
Sodium pyruvate	0.8 g
N-acetyl glucosamine	0.4 g

The above BSKII medium was filter sterilized and supplemented by the addition of 200 mL of autoclaved 7% gelatin and rabbit serum to give a final concentration of 6%.

to isolate spirochetes from arthropods. A nonantibiotic tube of culture medium was always run in parallel.

Attempts were made to isolate *B recurrentis* from blood and lice while in Ethiopia (1993). Samples were inoculated into both Kelly's and BSKII medium, which was incubated at 35°C until a color change occurred. These cultures were passaged into fresh medium. (Microscopic analysis was not done.) During the second visit, all cultures were blindly passaged every third day. On transfer of all culture tubes to the United Kingdom, all were analyzed by dark field microscopy and any containing motile spirochetes were inoculated into fresh culture medium. No incubator was available in Tanzania, hence all incubations were done at room temperature with all cultures blindly passaged every third day. On return to the United Kingdom, these were all analyzed using dark field microscopy.

RESULTS

Following the initial visit to Ethiopia, microscopic examination of culture tubes inoculated with blood samples from 15 patients with louse-borne relapsing fever revealed spirochetes. All with the exception of one, were nonmotile upon return to the United Kingdom. BSKII medium contained spirochetes from 5 patients while the Kelly's media were negative, and in the remainder of patients produced a much higher yield than the Kelly's media. Consequently, Kelly's media A and C were not pursued further. The motile spirochetes were grown from a blood sample drawn from a 24-year-old seaman who had been resident in Addis Ababa for two weeks prior to his admission to the Black Lion Hospital as part of the study of louse-borne relapsing fever.[18] He gave a three-day history of fevers, chills, headaches, dizziness, cough, chest pain, musculoskeletal aches, abdominal pain, anorexia, nausea, and vomiting. Upon investigation he

Table 2. Kelly's medium A.

Compound	Quantity per Liter
Sodium diphosphate	26.52 g
Sodium phosphate	1.03 g
Sodium chloride	1.2 g
Potassium chloride	0.85 g
Magnesium chloride	0.68 g
Glucose	12.75
Neopeptone*	5.95 g
Tryptone	2.55 g
Sodium pyruvate	1.06 g
Sodium citrate	0.47 g
N-acetylglucosamine	0.53 g
Bovine serum albumin fraction V	100 g
Sodium bicarbonate	1.5 g
Phenol red	0.03 g

** = Proteose peptone number 2 was used in the original medium by Kelly. The above was sterilized by passing through a 0.22μ filter then supplemented by the addition of 300 mL 7% autoclaved gelatin and sterile rabbit serum to give a final concentration of 6%. Medium was then aseptically dispensed.*

was found to have 14,000 spirochetes per μL of blood. This isolate successfully subcultured in BSKII and has now been passaged in vitro in excess of 70 times. **Figure 3** shows the spirochetal morphology of these cells. The isolate gave a growth yield of 10^7 organisms per mL and had an estimated generation time of 8-9 hours.[18] It has successfully been resuscitated from frozen cultures stored at -70°C on many occasions.

To maintain this isolate by in vitro subculture, best results were achieved when a large inoculum was used

Table 3. Kelly's medium C.

Compound	Quantity per Liter
Sodium chloride	13.8 g
Bovine serum albumin fraction V	44 g
Neopeptone*	13 g
Yeast extract†	2.64 g
Sodium hydrogen phosphate	0.2 g
Potassium hydrogen phosphate	0.12 g
Glucose	5.5 g
Calcium chloride	0.33 g
Sodium citrate (disodium salt)	0.34 g
Asparagine	0.11 g
Choline chloride	0.03 g
Sodium hydrogen carbonate	14 g
Ascorbic acid	1.1 g

**Proteose peptone was used by Kelly.*
†Yeastolate was used by Kelly.
The above was filter sterilized using 0.22μ filters, and supplemented by the addition of 214 mL/L of 10% autoclaved gelatin and sterile rabbit serum to give a final concentration of 6%.

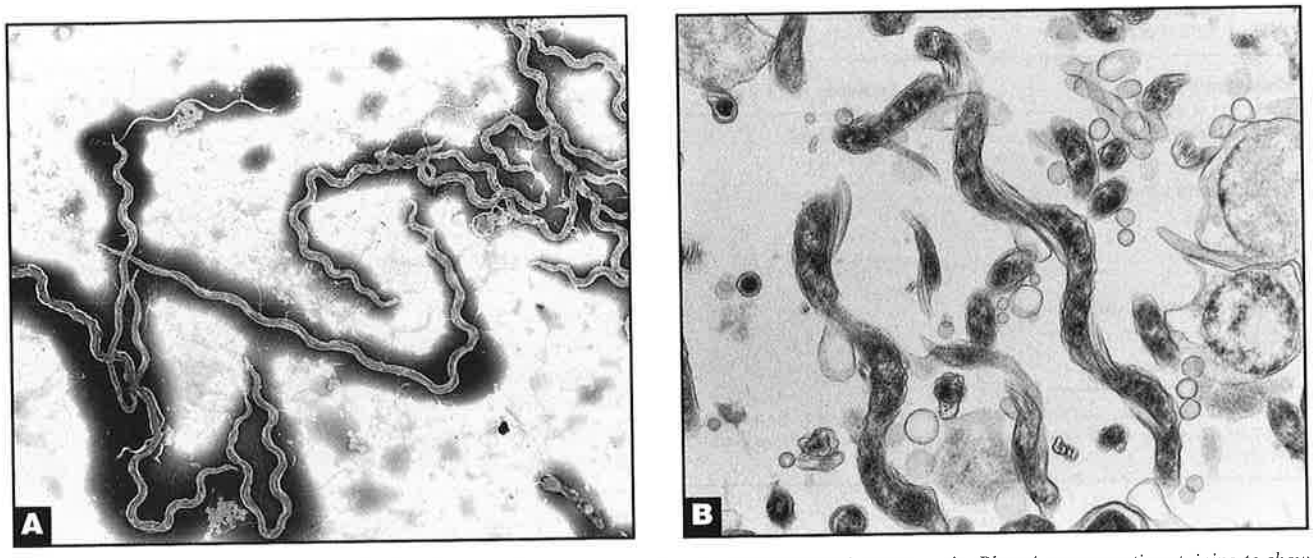

Figure 3. Low and high magnification electron microscopic pictures revealing the morphology of B recurrentis. *Plate A uses negative staining to show the spirochetal shape with tapering ends (magnification ×8800), while plate B is of thin section transmission electron microscopy to reveal more detailed morphology, especially the location of the periplasmic flagella in relation to the cell (magnification ×41,600).*

(approximately 0.5 mL of an actively growing culture) and regular subculture was done every three to four days. Once the spirochetes had become nonmotile, subculture was no longer possible. Multiplication was slow at 28°C, while temperatures of 34°C and 40°C gave excellent growth.

The culture attempts from lice were unsuccessful, with all attempts being overgrown by contaminants. No spirochetes were visible among the contaminants.

Following a second visit, 17 further cultivable isolates were obtained from a total of 34 patients with louse-borne relapsing fever.[19] This improved success rate was probably a result of blindly passaging into fresh BSKII medium every third day.

Similar methods were used during 1998 in Tanzania to isolate *B duttonii* from 12 patients with tick-borne relapsing fever.[20] Contamination problems were frequent as the blood samples were glass capillary specimens taken under poor conditions from malnourished children (age ranged from 4 months to 3 years old). Four viable isolates were brought back to the United Kingdom. A further isolate was recovered from repeating cultural work with room temperature-stored capillary bloods on return to the United Kingdom. One of the cultures was contaminated with a filamentous fungus, but was purified following passage through 100 μg/mL amphotericin B. The remaining isolates were purified by passage through the antibiotic-containing BSKII detailed in the Materials and Methods section.

O moubata ticks that were processed on return to the United Kingdom, failed to yield cultivable strains of *B duttonii.*

Preliminary characterization of these newly cultured isolates of relapsing fever *Borrelia* has previously been published.[19,20] The isolates of *B recurrentis* fell into six different groups based on their protein profiles (SDS-polyacrylamide electrophoresis), while the *B duttonii* gave four different profiles. Sequencing studies of their P66 protein and flagellin have shown isolates of each species to be conserved (unpublished findings).

DISCUSSION

Relapsing fever *Borrelia* have been largely unstudied as attempts to cultivate over the years have been plagued with problems. Cultivation of certain *Borrelial* strains became possible with the use of Kelly's media,[11,12] and was further improved by the introduction of BSK II medium.[14] Once cultivation of relapsing fever *Borreliae* became possible, the doors were opened allowing research into the mechanisms of antigenic variation, gene organization, and investigation into pathogenic mechanisms. Now that two of the most notorious members of this genus are cultivable in vitro, full investigation of these may also be pursued with the long-term goal of their eventual elimination.

The physiological requirements of these strains have yet to be fully determined. Similarly, the in vivo requirements of strains has yet to be established. *B recurrentis* are not easily adapted to growth in any alternative mammal other than the human host.[10] Only monkeys have successfully been used to propagate the organism and to produce a relapsing clinical picture similar to that seen in man.[21-23]

The serospecificity of *Borrelia* in each clinical relapse

is determined by their outer membrane proteins (collectively known as variable membrane proteins, vmp, with large members known as vlp and small as vsp). These serotypes differ in molecular weights of their major outer membrane protein, peptide maps, and reactivity with monoclonal antibodies. Initial studies with *B hermsii* identified 24 different serotypes using serospecific FITC-labelled antibodies[24] and two further serotypes were later described.[25] This group noted that seroconversions were not random but showed preferential switching to certain serotypes, especially serotype 7 in the first relapse.[24] Conversions were also demonstrated to occur in the absence of antibody in fortified Kelly's medium at a rate of 10^{-4} to 10^{-3} cells per generation and were frequently noted to contain mixtures of serotypes.[24] More recently, spontaneous variation has been described for *B turicatae* with two novel serotypes arising following 50 consecutive passages in vitro using BSKII.[26] It is possible that this too may be occurring among populations of spirochetes in this study. A less predominant band was observed in three of the five *B duttonii* and two of the *B recurrentis* isolates in the region of vsp outer membrane proteins (19-24 kda). These potential subpopulations have not been cloned or characterized as yet.

Prolonged in vitro culture of *B hermsii* has resulted in a culture-associated "C" serotype of *B hermsii*, now known as vsp33. Although this serotype was derived from serotype 7 followed by in vitro passage, it lacked antigenic relatedness to either type 7 or 21 and gave a molecular weight of 20 kda as opposed to 39 and 38 kda, respectively.[25] When this type is inoculated into mice, it persists for 2 to 3 passages before reverting to disease-associated serotypes.[25] This particular serotype shows only low frequency vmp switches. Additionally, expression site for vsp33 is different from that used for other vmp genes characterized to date.[27] A surprising finding was that this vsp showed greater homology with other vsp's of similar molecular weight and OspC of *B burgdorferi* than it did with other 35 to 39 kda vlp's derived from the same isolate.[28] A switch to this serotype can also be induced by decreasing the culture temperature of the spirochetes.[29]

A central role of the vmp lipoprotein in disease presentation has been documented for *B turicatae* infection in mice, with those expressing vmp type A producing neurological sequalae, while type B result in larger numbers of blood-borne spirochetes. Antigenic variation has again been identified in *B turicatae* where two different serotypes have been associated with very different clinical outcomes. In a mouse model infection with serotype vmpB results in a severe arthritic manifestation while serotype vmpA results in more extensive central nervous system involvement. The number of *Borreliae* present in joints or blood of those mice infected with serotype B was far in excess of that seen with serotype A suggesting a possible relationship between vmp serotype and disease severity.[30,31]

The major membrane lipoproteins of *B recurrentis*, like other members of this genus, are potent inducers of TNF-alpha, but also stimulate IL-1B, IL-6, and IL-12. These lipoproteins at concentrations of 10 to 20 μmol/L produce a TNF-α stimulation equivalent to 30 to 40 ng/mL of lipopolysaccharide (40 ng/mL LPS = 10 nmol/L).[32] A TNF-inducing toxin from strain A1 of *B recurrentis* is approximately half as potent as LPS on a molar basis. Comparison of TNF-inducing activity has been shown to vary by at least 50-fold depending on which size of *B recurrentis* vmp is used.[32] It is possible that different expression of vmp may influence the severity of clinical disease seen in *B recurrentis* and *B duttonii* infection.

The use of BSKII to propagate isolates of relapsing fever spirochetes may subject the *Borrelia* to a selective pressure. This has been reported to occur for the closely related *B burgdorferi* when genetic diversity among cultivated spirochetes has been compared with those characterized directly from ticks.[33] This could be further investigated for relapsing fever by analyzing blood samples taken from patients using molecular techniques to characterize the spirochetes present. If indeed types are found that are not represented among those seen in vitro, selective pressure may indeed be a factor.

The ability to cultivate *B recurrentis* and *B duttonii* should now make it possible for these fastidious organisms to be characterized, their full pathological potential to be investigated, and elimination strategies assessed.

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Ehrlichiosis in Children

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ABSTRACT

Ehrlichiosis is azoonosis caused by intracellular bacteria that invade circulating blood cells and are associated with arthropods and helminths. On the basis of ribosomal DNA sequencing, *Ehrlichia* species have been classified among the α proteobacteria close to Rickettsiae. To date, only *Ehrlichia chaffeensis,* the agent of Human Monocytic Ehrlichiosis (HME) and Human Granulocytic Ehrlichiosis (HGE) have been associated with disease in children.

The two diseases cause similar clinical manifestations, with mainly fever, flu-like symptoms, and cytopenia. HME has been reported only in the United States and is transmitted by the tick *Amblyomma americanus,* which is not found in Europe. Conversely, HGE, transmitted by ticks from the genus Ixodes, has been observed in Europe as well as in the United States and should be suspected in the same circumstances than those suggesting Lyme disease.

Specific diagnosis relies mainly on serology. Tetracyclines are the first-choice antibiotic for treating ehrlichiosis in children older than 9 years. Rifampin may be useful for younger children.

Key words: Ehrlichiosis, cytopenia, rickettsia, anthropo-zoonosis

INTRODUCTION

Ehrlichiosis is a group of emerging anthropo-zoonosis caused by small, obligate intracellular bacteria. Their incidence has been increasing regularly in adult and in children, as shown by the growing number of reported clinical observations.[1-10] This article focuses on the epidemiologic, clinical, and biological features as well as treatment and prevention of the ehrlichiosis in children.

MICROBIOLOGY

Bacteriology. Ehrlichia are small, obligate intracellular gram-negative bacteria, appearing violine with May-Grünwald-Giemsa or Diff Quick. They replicate into circulating blood cells (monocytes, granulocytes, red blood cells, platelets) and sometimes in the vascular endothelial cells.

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Phylogeny. Analysis of 16S rDNA sequence made possible the classification of Ehrlichia within the alpha group of *Proteobacteria* close to the genus *Rickettsia.* This approach has been used also to divide the genus Ehrlichia into four genogroups (**Table).** The oldest group, *Ehrlichia* sensu stricto, includes *E sennetsu*, etiologic agent of the Japanese human ehrlichiosis that has not been reported for 25 years, *E risticii, Neorickettsia elokominica,* and *Neorickettsia helminthoeca.* The second group is *Wolbachia,* which are associated with insects, helminths, and shellfish. The third group is the *Cowdria* group that includes *Cowdria ruminantium, E canis, E chaffeensis, E ewingii, E muris,* and the agent of human monocytic ehrlichiosis of Venezuela whose pathogenicity has not been demonstrated. Lastly, the fourth group, that of *Anaplasma*, also contains *E phagocytophila, E equi, E platys* as well as the agent of the human granulocytic ehrlichiosis. Three species are currently involved in human pathology: the agent of monocytic human ehrlichiosis (*E chaffeensis*), the agent of granulocytic human ehrlichiosis, and *E ewingii.*[11] To date, however, only monocytic human ehrlichiosis and granulocytic human ehrlichiosis were observed in children.

Pathogenesis. Ehrlichia are intracellular parasites that

Table. Characteristics of the main **Ehrlichia** *species known as human or animal pathogens.*

Genogroup	Species	Host	Disease	Target Cells in vivo	Vector	Distribution
1	*E sennetsu*	Human	Glandular fever	Monocytes-macrophages	Helminthes ?	Japan
1	Agent SF	Metacercaire of *Stellanchasmus falcatus*	Fever in dogs, lymphadenitis in mice	Mononuclear cells	*Stellanchasmus falcatus* (helminthe)	Japan
1	*N helminthoeca*	Dog and Canidae	Salmon poisoning syndrome	Macrophages	Helminthes	California, Oregon, Idaho, Washington
1	*E risticii*	Horse	Equine Monocytic Ehrlichiosis or Potomac Horse Fever	Monocytes-macrophages, epithelial intestinal cells	Helminthes ?	USA and Europe
2	*Wolbachia sp*	Insect	Fertile incompatibility, parthenogenesis	?	*Culex pipiens* (mosquito)	?
3	*C ruminantium*	Goat, sheep, cattle	Pericarditis	Endothelial cells	*Amblyomma spp* (tick)	Afrique et Caraïbes
3	*E canis*	Dog	Canine Ehrlichiosis or Tropical canine pancytopenia	Monocytes-macrophages	*Rhipicephalus sanguineus* (tick)	Ubiquist
3	*E ewingii*	Dog	Canine Granulocytic Ehrlichiosis	Granulocytes	?	USA
3	Human Venezuelian Ehrlichiosis	Human	Asymptomatic	Monocytes	?	Venezuela
3	WSU86-1044	Cattle	?	?	?	Washington
3	*E muris*	Mouse	?	Monocytes	?	Japan
4	*Anaplasma sp*	Cattle	Haemolytic anemia	Erythrocytes	*Dermacentor andersonii* et *D variabilis* (ticks)	Southern Europe, Africa, America, Asia, former USSR
4	Human Granulocytic Ehrlichiosis Agent	Sheep, horse, dog, deer	Human Granulocytic Ehrlichiosis	Granulocytes	*Ixodes ricinus* and *I scapularis* (ticks)	USA, Switzerland, Italy, Sweden, United Kingdom, Norway, France
4	*E phagocytophila*	Cattle, sheep, buffalo	Tick-borne fever	Granulocytes	*I ricinus* (ticks)	United Kingdom, Europe
4	*E equi*	Horse	Equine Ehrlichiosis	Granulocytes	*Ixodes spp* (ticks)	USA and Europe
4	*E platys*	Dog	Cyclic canine thrombocytopenia	Platelets	?	USA
Not classified	*E bovis, E ovina*	Cattle, sheep	Bovine Ehrlichiosis, Ovine Ehrlichiosis	Monocytes-macrophages	*Hyalomma rhipicephalus, H bursa* (ticks)	Middle East, Africa, Sri Lanka

penetrate in the host cell through phagocytosis and multiply within phagosomes. They form intracytoplasmic colonies limited by a membrane called morula. The lysis of the phagosome releases the bacteria and allows their propagation to new cells.[12] *Ehrlichia* infects specifically the cells of the immune system, and each species of *Ehrlichia* has a particular cellular tropism **(Table 1)**. During the acute phase of the disease, the bacteria can be found within the circulating cells (monocytes for *E chaffeensis*, granulocytes for the agent of human granulocytic ehrlichiosis). *E chaffeensis,* infection is found also in macrophages of the monohistiocytaire system (liver, spleen, lymph nodes, bone marrow, central nervous system), where it frequently induces inflammatory granuloma with no necrosis.[13]

Human Monocytic Ehrlichiosis (HME)

Epidemiology. To date, 50 cases of HME have been documented in children from a total of 500 cases reported since 1986.[1-5,7-10] All confirmed cases were observed in the United States. The mean age in children was 7 years (range 7 months to 14 years) with 57% being males. Most children were living in rural areas where recreational and occupational activities in forests appear to be a risk factor for the infection. In 80% of the cases, infection occurred in May and June.[14] Concomitant HME and significant underlying diseases have been described in adults[15] Of the 3 children who presented with an associated disease, one had Down Syndrome,[1] one had β-thalassaemia, the third had renal transplantation 6 weeks earlier.[5] The majority of the children had a positive history for a tick bite 2 to 21 days before the beginning of the symptoms.[6] The responsible tick, *Amblyomma americanum*, has never been identified in Europe, explaining the absence of this disease on this continent.[16]

Clinical Presentation. HME has a wide clinical spectrum, from inapparent infection to severe life-threatening disease.[5,10] Fever is almost constant.[14] A maculopapular or sometimes petechial rash, distributed on the trunk or extremities, is noted in 66% of the children whereas it is found only in 36% to 47% of the adults. Headache and myalgias are present in 63% of the patients. Gastrointestinal symptoms (nausea, anorexia) are reported in 57% of the patients, and hepatosplenomegaly in 41%.[14] Other reported abnormalities include nuchal rigidity, photophobia or conjunctivitis, arthralgia,[3,4,7] cervical adenopathy.[3,5,8,9] The frequency of severe forms in children is difficult to estimate; in one serie, 25% of all patients with HME required intensive care therapy (hemodialysis for acute renal failure and prolonged mechanical ventilation). Long-term neurologic sequelae have been recognized in two children: one presenting a bilaeral foot drop and the other a speech impediment.[5,10] To date, only one death was caused by HME. The death resulted from a nosocomial pneumopathy caused by *Stenotrophomonas maltophilia* after prolonged hospitalization.[10]

Biological Features. Elevated serum aminotransferase levels, thrombocytopenia (<150.000/mm^3) and lymphopenia (<1500/mm^3) are seen in 80% of the children and are more pronounced at 7 days of illness. Anemia is noted in about 40% of HME in children only and is usually not severe. A hyponatremia lower than 135 mmol/L was found in 65% of the children.[14] In rare cases where cerebrospinal fluid was examined, a mild pleiocytosis (median 100/mm^3) with a lymphocytic predominance was shown.[4,5,7-10]

Diagnosis. In an endemic area (southeast of the United States), patients who present during the summer with unexplained fever, leucopenia, thrombocytopenia, hepatitis, and a history of tick bite (but children may not mention the tick attachment) should be considered to be at risk for HME. The main differential diagnosis is Rocky Mountain Spotted Fever. A clinically compatible history with a fourfold or greater rise in the indirect immunofluorescent antibody titer to *E chaffeensis* (with a minimum titer of 64) is required for case definition for HME.[15] Direct examination of the peripheral blood smear looking for morulae in monocytes has been a very insensitive poor method for establishing the diagnosis. Examination of the monocytes obtained by bone marrow aspiration appeared as more sensitive.[2,5] Isolation of *E chaffeensis* from blood culture was difficult and required at least a 30-day incubation period. It is therefore unlikely to be useful to clinicians.[17] PCR-amplification of the 16S rRNA encoding gene of *Ehrlichia* from the blood of patients or from infected ticks appeared to be promising.[18] The sensitivity approaches 90%.[14] Furthermore, molecular methods allowed the identification of *E chaffeensis* in some patients with negative serologic testing.[18]

Human Granulocytic Ehrlichiosis (HGE)

Epidemiology. To date, no epidemiological data concerning children specifically are available for this newly-discovered disease. Since 1994, 43 cases were reported in the literature, including 2 in children. [19,20] This small number could be related to the recent discovery of the affection, and the proportion of children is the same as that observed in HME. In adults, the median age (45 years) and the male predominance were similar to those described for HME.[14,20] Risk factors for transmission were the same as those observed for Lyme disease: recreational or occupational activities in forests and exposure to wild animals (deers). Most of the cases occurred from September to April. The disease was observed in the United States and in Europe.[14,21-24] A history of a tick bite before the beginning of disease was reported by 90% of

the patients.[19] Ticks of the genus Ixodes, particularly *I scapularis* in the United States and *I ricinus* in Europe, appeared as the main vectors of HGE.[25] *I scapularis* and *I ricinus* are also the vectors of *Borrelia burgdorferi*. A case of coinfection with HGE and Lyme borreliosis has been reported in a child.[7] Perinatal transmission of the agent of HGE was recently described, suggesting potential transplacental transmission of the micro-organism.[20]

Clinical Presentation. Since data available about HGE and children are limited, the clinical features of this illness are based on observations made in adults. The symptoms of HGE appear to be similar to those of HME, with fever, myalgias, sweats, and headache.[26] However, unlike HME, the clinical examination is generally normal.[19] Asymptomatic infections have been described. The mortality rate with HGE ranges from 5% to 10%, but severe infection has never been reported in children.[19] In adults, deaths were related to development of secondary pulmonary infections.[27]

Biological Features. Leukopenia and thrombocytopenia are found in 58% and 83% of the patients respectively.[14] Mildly elevated serum aminotransferase and lactate deshydrogenase levels and a moderate inflammatory syndrome were noted in 90% of the patients.[19] Anemia (50% of the patients), lymphopenia, increased percentage of neutrophils, and the presence of morulae within the neutrophils of the perpheral blood have been identified as pejorative prognostic factors.[19] A mildly elevated serum creatinine level (<115 mmol/L) was found in 70% of the patients.

Diagnosis. HGE should be suspected in patients who are seen during the summer with a history of fever following a tick bite in an endemic area for Lyme disease (Northeastern United States, Northeastern Europe): of 228 patients corresponding to these criteria, 41 (21%) were infected with HGE.[19] Diagnosis of HGE is based on an indirect immunofluorescence antibody assay. In the presence of a clinically compatible history, seroconversion or four-fold increase in HGE-specific antibody titers between an acute and convalescent serum sample are required to confirm the diagnosis.[15] Direct examination of blood smears stained by Giemsa alcohol or Diff Quick demonstrates morulae in the cytoplasm of peripheral blood neutrophils in 80% of the patients.[19] Caution should be used in interpreting these results because of the low sensitivity of direct examination: a clinician should never discount the diagnosis of HGE in a patient because the peripheral smear does not demonstrate the characteristic morulae. Isolation of the HGE agent can be performed in specialized laboratories using coculture of blood sample with tissue cell line. Polymerase chain reaction-amplification of the 16S ribosomal RNA encoding gene is possible from peripheral blood sample. This molecular tool has been shown to have a sensitivity of 86% and a specificity of 100% and allows therefore an early diagnosis.[28]

Treatment

According to clinical data and in vitro susceptibility studies, the first choice antibiotic for treating HME and HGE in children older than 9 years should be doxycycline administred at 4 mg/kg per day twice daily with a maximum dose of 200 mg/j for 7 to 10 days.[5,14,29] Although doxycycline is theoretically contraindicated in children younger than 9 years because of possible staining of the teeth, this antibiotic has been chosen by several physicians for the treatment of any patient, regardless of age, with symptomatic ehrlichiosis. This choice is supported by the best efficacy of doxycycline and by the knowledge that the staining of the teeth by the tetracyclines seems to have been totally dose related.[5,30] Rifampin may be an acceptable alternative agent, as suggested by in vitro susceptibility data and by its clinical effectiveness in a pregnant woman with HGE.[31,32]

Prevention

Avoidance of tick-infested areas is the first line of defense against ehrlichiosis. Children going in wooded, tick-infested, areas should wear protective clothing and even a hat. If long-sleeved shirts or long pants are not practical, exposed area of the skin should be covered with insect repellents containing N-N-diethyl-M-toluamide (DEET). Because systemic reactions to DEET can occur when concentrations are too high, repellents should be used carefully in young children and chronic readministration should be avoided. After returning from wooded areas, children should be inspected, with emphasis on body areas containing hairs. If ticks are detected, they should be removed after skin disinfection. There currently is no justification for the use of antimicrobial prophylaxy after a tick bite.[14]

NOTE

Since this article has been submitted, 449 cases of HGE have been serologically identified by the CDC.[33]

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From Discovery to Clinical Implications: *Borrelia valaisiana*

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ABSTRACT

From the discovery of the isolate VS116 in Valais, Switzerland, to the time when the name *Borrelia valaisiana* was attributed to it, many scientists have been involved in characterization of this microorganism. New data on the phenotypic characterization and aspects of the various genetic methods used to type this *Borrelia* are reviewed. Epidemiological data, geographical distribution, and vector-host relationships are described. Finally, the possible role of *B valaisiana* in human cases of Lyme borreliosis is discussed.

Key words: lyme, *borrelia valaisiana*, group VS116, group M19

HISTORY OF THE DISCOVERY

The discovery of the spirochete responsible for Lyme disease in 1982 by Burgdorfer et al[1] in the United States sparked renewed interest in tick-borne diseases throughout the world. Indeed, the following year, a similar spirochete was isolated from Swiss *Ixodes ricinus*.[2,3] We know now that the spirochete originally named *Borrelia burgdorferi* covers all temperate regions of the northern hemisphere. In fact, it was found to be restricted to the distribution of the tick vectors belonging to the *I ricinus* complex. A few years later, it appeared that the clinical symptoms of Lyme borreliosis in somewhat different from those observed in the United States.[4] Similarly, the *B burgdorferi* isolates in Europe appeared much more heterogeneous than the isolates in the United States.[5,6]

In Switzerland and in other European countries where Lyme borreliosis is endemic, the tick *I ricinus* is the main vector. In 1986, we collected ticks in various regions of the state of Valais **(Figure 1)** by dragging white flannel through bush and low vegetation. In the laboratory, each adult specimen was dissected and examined for spirochetes. Pieces of midgut tissues were removed for the preparation of smears that were later stained by indirect immunofluorescence to detect the presence of *Borrelia*. Additional pieces of midgut were removed and inoculated into BSK II medium for isolation of the organisms. Approximately 30% of the ticks proved to be infected.[7,8] Of the first BSK II cultures, 21 isolates of *B burgdorferi* were recovered. Results of the protein profiles after gel electrophoresis provided evidence of important variations in antigenic compositions among isolates from the state of Valais. Monoclonal antibodies were raised to one of these atypical isolates. One monoclonal antibody, D6, showed reactivity to a very low molecular mass protein, of about 12 kda, and identified only the isolates with a particular OspA pattern. From this observation, we proposed a classification of the *B burgdorferi* isolates, leading to 4 groups.[9] Collaborative studies[10,11] confirmed that our classification was correct for groups 1 to 3. Groups 1 and 3 represented isolates, further named by Baranton et al,[12] *B burgdorferi* sensu stricto and *Borrelia garinii*, respectively. The isolate VS461, representative of our group 2, was chosen as the reference strain for *Borrelia afzelii*.[13] The group 4, exemplified by the isolate VS116, was set aside until it was recognized as a new genetic group.[14] In the meantime, a group M19 was identified among Dutch *I ricinus* in The Netherlands. Group M19 was later considered identical to the previously described

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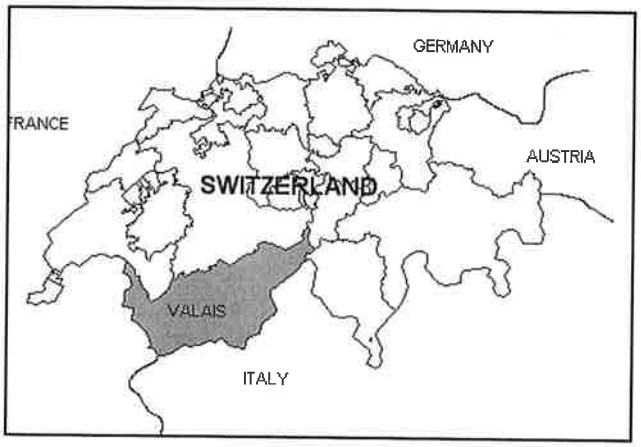

Figure 1. Map of Switzerland (Valais) with surrounding countries.

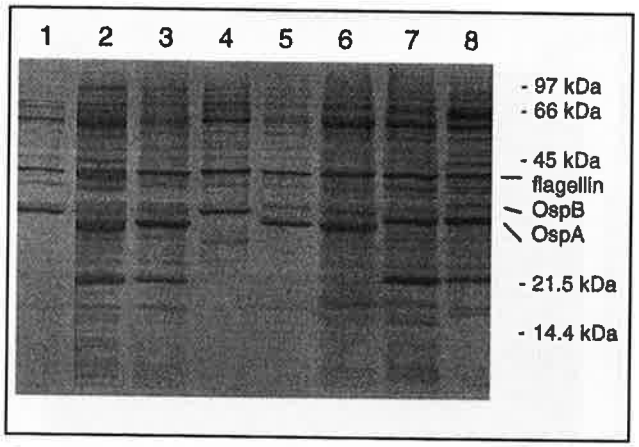

Figure 2. SDS-page of whole cells of B burgdorferi *isolates stained with Coomassie blue. Lanes 1 to 5*—B valaisiana*: VS116, NE231, Frank, UK, M7. Lane 6*—B burgdorferi: *sensu stricto, reference strain B31. Lane 7* —B garinii: *reference strain 20047. Lane 8*—B afzelii: *reference strain VS461. Molecular mass standards in kilodaltons (kda) are indicated on the right.*

group VS116.[15] Finally, several years later, the name *B valaisiana* was given to the isolates belonging to M19 and VS116 groups.[16]

PHENOTYPIC CHARACTERIZATION

OspA Profiles of *B valaisiana* Isolates

Phenotypic analysis of European *B burgdorferi* sensu lato isolates has demonstrated antigenic and structural heterogeneity of the OspA protein, one of their major outer membrane lipoproteins.[9] OspA has therefore been used for serotyping and serological diagnosis, and for vaccine development.[17-20] The apparent molecular mass of OspA ranges from 32 to 34 kda among the different isolates of *B valaisiana* (**Figure 2**). Phenotypic identification of most *B valaisiana* isolates can be achieved with the species-specific monoclonal antibody A116k reactive to the OspA.[21] However, four isolates (NE231, Frank, M7, and M53) of 24, presenting an OspA with a molecular mass of 32 kda, were unreactive. Recently, three *Borrelia* strains (5MT, 9MT, and 10MT) isolated from *Ixodes nipponensis* in Korea were classified as *B valaisiana.*[22] Strains 5MT and 10MT presented an OspA of 32 kda reactive with the anti-OspA monoclonal antibody P31c. Further analysis is needed to confirm the specificity of these two monoclonal antibodies for the two subgroups of *B valaisiana* isolates.

Preliminary results of OspA gene sequences confirmed the existence of two groups among *B valaisiana* isolates. Group I containing strains VS116, UK, and AmSO1, presents a molecular mass of 34 kda for OspA, and the second group (NE231, Frank, M7, and M53) presents an OspA of 32 kda.[21]

Description of *B valaisiana* Proteins by 2D-Electrophoresis

Since its introduction in the mid-seventies, high resolution 2-D polyacrylamide gel electrophoresis has been used for many types of proteins, including membrane proteins. Isoelectric focusing, however, was achieved with an unstable pH gradient at the time of the first electrophoresis. The undefined chemical composition of carrier ampholytes and the long period of separation did not assure the stability of the pH gradients. The improvement of protein solubility allowed for better results.[23,24] The technique used in this study was mainly based on these recent improvements.

To determine the molecular mass and the isoelectric point of *Borrelia* proteins, human plasma proteins were simultaneously submitted to 2D-electrophoresis with *B valaisiana* proteins (VS116). The protein map of *B valaisiana* (**Figure 3**) was established with the Melanie program (BioRad, Zursach-CH).[25] Results are presented in **Table 1.** Some proteins of *B valaisiana* were identified and confirmed with monoclonal antibodies directed to 93 kda, 66 kda, 62 kda, Flagellin, 39 kda, OspA, and OspC. A first comparison between *B burgdorferi* sensu stricto (VS215), *B garinii* (VS102), *B afzelii* (VS461), and *B valaisiana* (VS116) was realized. Differences were mainly observed on 93 kda, 61 kda, and 47 kda proteins, and OspA and OspC among different species. The 37 kda and the OspC proteins of *B valaisiana* (VS116) presented a better focalization than proteins of other species. These

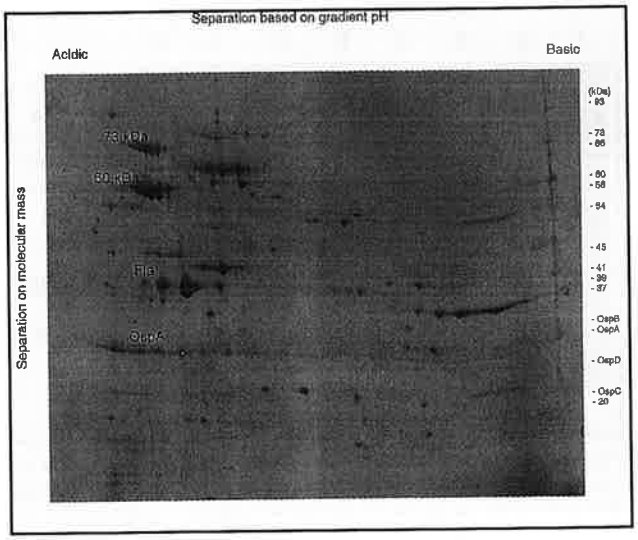

*Figure 3. 2D-electrophoresis of VS116 (*B valaisiàna, *reference strain). Gel stained with Coomassie blue. Molecular mass in kilodaltons (kda) are indicated on the right.*

results were part of a preliminary study.[21] More strains and species need to be tested to confirm inter- and intra-species reproducibility.

GENOTYPIC CHARACTERIZATION

DNA–DNA Reassociation

DNA–DNA reassociation allows the distinction of levels of DNA homology. This method is used to examine the relationship between closely related taxa. It represents one of the best applicable procedures for bacterial taxonomy.[26] The phylogenetic definition of a species would include all strains with approximately 70% or greater DNA–DNA relatedness and with 5°C or less ΔT_m. Percentage of DNA homology between *B valaisiana* strains and different species is summarized in **Table 2.**[14]

Pulse-Field Gel Electrophoresis

First described in 1984, Pulse-Field Gel Electrophoresis (PFGE) is a molecular typing technique to examine the chromosomal DNA profile of many microorganisms[27] that also proved to be an efficient method for typing *Borrelia*.[28,29] After digestion with a restriction enzyme having relatively few recognition sites, the bacterial genomic DNA is separated by PFGE. Discrimination of the species and among isolates is based on the large restriction fragment length polymorphism (LRFLP) of the chromosomal DNAs. After digestion with *Mlu*I, the LRFLP of *B valaisiana* is characterized by two bands of 340 and 90 kb.[30]

Table 1. Protein isoelectric points and molecular mass of VS116 (B valaisiana, reference strain).[21]

Proteins	Isoelectric Point
93 kda	5.0
78-80 kda	6.3-6.5
73 kda	5.1-5.4
66 kda	6.1-6.5
61 kda	5.7/6.1/6.5
60 kda	5.1-5.4/6.2/6.5
59 kda	5.2-5.5/6.5-6.6
57 kda	5.0/6.3
54-55 kda	5.0/6.2-6.4/6.6-6.7
47 kda	5.0-5.5/6.6
45 kda	5.9-6.5
41 kda	5.1/5.3/5.5/6.3/6.7-6.8
40 kda	5.1/5.5
39 kda	5.2-5.3
37 kda	6.5/6.8/7.1/7.7/8.0
34 kda	6.8
OspA	5.0/5.1/5.2/5.5/5.9/6.4
OspD	6.5/6.6/6.7/6.8
OspC	5.0/5.8/6.9
20 kda	6.7

Ribotyping

rRNA restriction analysis, or ribotyping, has been used to characterize *Borrelia* species.[31] Phylogenetic analysis with this method is based on the profiles obtained by restriction fragment patterns of chromosomal DNA digested with restricted enzymes and hybridized with a probe derived from a highly conserved rRNA. *B valaisiana* DNA restriction pattern after digestion by *Eco*RV is characterized by three fragments (6.9, 3.2, and 1.4 kb) after hybridization with a16S-23S cDNA probe **(Table 3).** With the same probe, the result of the digestion by *Hin*dIII showed four fragments (2.1, 1.2, 0.8, and 0.6 kb).[16,32] *Eco*RV digest of *B valaisiana* (M19 group) revealed a unique fragment around 3 kb when hybridized with a flagellum probe.[32] *B valaisiana* strains isolated from *I nipponensis* in Korea presented different RFLPs by hybridization with a flagellum probe.[22]

Species-Specific Polymerase Chain Reaction

Polymerase chain reaction (PCR) can be performed for the identification of the Lyme borreliosis spirochetes using species-specific primers. This approach has been used to differentiate the three human pathogenic species, *B burgdorferi* ss., *B garinii,* and *B afzelii,*[33] and *B valaisiana.*[21,34] Primers directed to a conserved region of 16S rRNA[34] or to OspA gene[21] have been designed to specifically amplify *B valaisiana.* The specificity of the PCR assay to OspA gene was evaluated on 113 *B*

Table 2. Percentage of DNA homology between **B valaisiana** *isolates and different species*[14] *(used with permission).*

% Homology with Strain	VS116	UK
B burgdorferi (B31)	54	65
B garinii (20047)	56	63
B afzelii (VS461)	64	70
B japonica (IKA2)	58	nt
B valaisiana (VS116)	100	93
B lusitaniae (PotiB2)	nt	60
B andersoni (21123)	60	nt
B bissettii (DN127)	46	nt

nt: non-tested

Table 3. **B valaisiana** *DNA restriction pattern after digestion by various restriction enzymes.*

Isolates	Gene Probe	Restriction Enzymes	Fragments	References
VS116	16S-23S	*Eco*RV	6.9, 3.2, 1.4 kb	(16)
VS116	16S-23S	*Hin*dIII	2.1, 1.2, 0.8, 0.6 kb	(16)
M19	*Fla*	*Eco*RV	one specific band around 3 kb	(32)
VS116	*Fla*	*Dde*I	188, 135, 117, 90, 33, 21 kb	(22)
5MT	*Fla*	*Dde*I	252, 188, 90, 33, 22 kb	(22)
10MT	*Fla*	*Dde*I	221, 135, 118, 90, 21 kb	(22)

burgdorferi sensu lato strains and 14 unrelated bacterial species. Cross-amplification was observed with three *B garinii* (SO1, BITS, and VS711), one *Borrelia lusitaniae* (BR41), and one *B afzelii* (DK3) strains. The specificity was therefore 94%. None of the 14 unrelated bacteria showed amplification with these primers. Twenty out of the 24 *B valaisiana* strains were amplified showing a fragment of 268 bp. Four isolates, NE231, Frank, M7, and M53, were not amplified with these primers. Sequencing of the OspA gene of *B valaisiana* isolates confirmed the presence of two distinct groups among this genospecies (see below).

Restriction Length Polymorphism of PCR Products

The organization of the rRNA genes in *Borrelia* strains associated with Lyme borreliosis is currently unique among known bacteria. The *rrn* cluster of most *B burgdorferi* sensu lato isolates contains a single copy of 16S rRNA (rrs) and tandem repeated 23S rRNA (*rrlA* and *rrlB*) and 5S rRNA (*rrfA* and *rrfB*). The rDNA gene cluster is located at the center of the linear chromosome and is arranged in the following order: *rrs-rrlA-rrfA-rrlB-rrfB*.[35]

PCR amplification of the spacer region located between the *B burgdorferi* sensu lato *rrf* and *rrl* genes generated a fragment that was 226 to 266 bp long, depending on the genospecies. After cleavage by *Mse*I, *B burgdorferi* sl strains were classified into 13 different patterns, designated patterns A to M. The size of VS116 *B valaisiana* amplicons was 255 bp and fell into pattern F, presenting four fragments (175, 50, 23, and 7 bp).[14] After cleavage by *Dra*I, *B burgdorferi* sl strains were classified into 12 different patterns. VS116 strain of *B valaisiana* fell into pattern B", presenting two fragments, 206 and 49 bp.[14] *B valaisiana* strains isolated from *I nipponensis* in Korea presented divergences in size of amplicons and restriction fragments from *B valaisiana* strains isolated from *I ricinus*.[22] The size of Am501 amplicons was 249 bp that fell after digestion with *Mse*I into pattern Q, presenting four fragments (169, 51, 23, and 6 bp).[22] Size of amplicons of strains 5MT and 10MT was 254 bp. They present, after digestion by MseI, five fragments (107, 59, 43, 24, and 22 bp) and four fragments (150, 58, 24, and 22 kb), respectively. The size of amplicons of strains 5MT, 9MT, and 10MT were 254 bp. They present, after digestion by *Dra*I, two fragments (173 and 81 kb).

Species of *B burgdorferi* sl can be distinguished from each other on the basis of their *Bfa*I restriction patterns of *rrs* amplicons.[36]

DNA Sequence Analysis

Whole DNA–DNA reassociation analysis is used to elaborate the phylogenetic relationship of *B burgdorferi* sl. DNA sequence analysis of some highly conserved gene loci can be a suitable alternative method to study taxonomy and epidemiology. For example, *rrs* and *fla*, have been used for this purpose with *B valaisiana*. Ribosomial sequences from various *B valaisiana* strains have been determined and are now available from the GenBank database. Lists of accession numbers are presented in **Tables 4** and **5.** Homology results of *B valaisiana* strains are presented in **Table 6.** The *fla* gene, currently known as *flaB*, encodes a 41 kda flagellin protein and is located on the linear chromosome.[37] A list of accession numbers is presented in **Table 7.**

The *ospA* gene, located on a 49- to 57 kb linear plasmid, is present in almost all *B burgdorferi sl* isolates. Sequence analysis of *ospA* gene of *B valaisiana* showed heterogeneity and revealed major subgroups among the species. Usually, the clustering of *Borrelia* strains in the phylogenetic tree, based on the sequence of *ospA* and its predicted amino acid sequence, is in agreement with the classification based on the sequence analysis of conserved chromosomal genes such as *rrs*, *p93*, and *fla*, as well as with data obtained by PFGE and RAPD fingerprinting. A recent study of eight *B valaisiana* isolates, however, showed that *ospA* sequence analysis might not be appropriate for

Table 4. List of accession number of rrs *gene sequences available on GenBank Database.*

Isolates of *B valaisiana*	*n°* Accession	References
VS116	X98232	(36)
UK	X98233	(36)
AM501	D67021	(36)
5MT	U44938	GenBank
9MT	L39080	GenBank

Table 5. List of accession number of 5S-23S rRNA intergenic spacer sequences available on GenBank DataBase.

Isolates of *B valaisiana*	*n°* Accession	References
VS116	L30134	(14)
UK	L30133	(14)
Am501	D84402	(77)
5MT	AB013914	(22)
10MT	AB013915	(22)

species identification of *B burgdorferi* sl. The list of accession numbers is presented in **Table 8.** Sequence identity matrix of partial sequences (819 bp) of *ospA* genes were described by Masuzawa[22] and are summarized in **Table 9.**

The conserved amino-terminal of the OspC protein revealed a species-specific motif. However, among *B valaisiana* strains, the complete *ospC* gene seemed to be transferred from *B afzelii*. Therefore, it is usually inconclusive for assignment of an isolate to a specific *Borrelia* species because of the overall high variability of *ospC* sequences.

Multilocus Enzyme Electrophoresis

Although based on electrophoretic migration of enzymes, Multilocus Enzyme Electrophoresis (MLEE) is a powerful phylogenetic method. Indeed, it reveals the polymorphism of genes encoding housekeeping enzymes of bacteria. Based on a significant number of genes, the MLEE can determine the genetic relatedness of isolates in a particular bacterial population and is also able to identify genomic groups. For the method, bacterial lysates are subjected to electrophoresis in starch gels under nondenaturing conditions. Enzymes are chosen for demonstrable and interpretable activities. Choice of buffers and pH buffers are also important for performance of the method. The electrophoretic mobility of metabolic enzymes is determined after specific staining. Each electromorph is equated with an allele at the corresponding enzyme genetic locus. Thus, by associating each isolate with an electrophoretic type (ET), MLEE allows the differentiation of isolates by marking them with significant characteristics.

MLEE was used to analyze the population genetics of 50 *B burgdorferi* sl isolates in 1992.[11] At that time, the isolate VS116 was not clearly differentiated from *B burgdorferi* ss. Later, this method was performed with a slightly modified panel of enzymes to determine the overall genetic polymorphism of 54 *B burgdorferi* sl isolates from different regions of the world and 3 additional relapsing fever Borreliae. Cluster analysis of a matrix of genetic distances for pairs of ETs revealed 11 divisions separated from each other at a genetic distance greater than 0.65 *B valaisiana* corresponding to division V.[38] This method, however, is labor-intensive, mainly because large quantities of each isolate have to be grown to obtain enough lysate for MLEE analysis.

Phylogenetic Position of *B valaisiana*

Clusters containing *B burgdorferi* ss, *B garinii, B afzelii, B japonica, B lusitaniae,* and *B valaisiana* were clearly differentiated on the basis of the hbb gene analysis. The genomic position of *B valaisiana* was in an independent branch, close to *B garinii* and *B japonica.*[39,40] A phylogenetic analysis of *Borrelia* species based on the flagellin gene sequences also situated the genomic position of *B valaisiana* in an independent branch, close to *B japonica.*[41] A phylogenetic analysis of *Borrelia* species based on the 16S rRNA gene described the *B valaisiana* species as closely related to the *Borrelia* species responsible for Lyme borreliosis rather than to other *Borrelia* species responsible for recurrent fever, such as *Borrelia lonestari, Borrelia miyamotoi, Borrelia anserina, Borrelia duttoni,* and *B hermsii.*[16]

The phylogenetic tree constructions, based on the amino acid sequence similarity matrix of highly variable proteins, are not reliable. For example, the *B valaisiana* isolates VS116, M19, and M53 constituted an independent branch with a construction based on the OspC protein. However, isolates M7 and UK fell into the clusters of *B afzelii*, and the isolates Ar-2, M52, and M49 fell into the clusters of *B garinii*, suggesting possible lateral transfers and recombinations.[42]

EPIDEMIOLOGICAL FEATURES OF *B VALAISIANA*

Geographic Distribution of Different *Borrelia* Species

B valaisiana has been recovered in several European countries and in Asia. This genospecies was mainly detected by PCR in ticks from Italy,[43] Croatia,[44-46] Russia,[47] Germany,[34,45,48] England,[45,48,49] Ireland,[45,48,50,51] and The Netherlands.[32,45,48,52-54] Currently, about 30 *B*

Table 6. Percent sequence similarity values of rrs *gene*[36] *(used with permission).*

Borrelia Species (isolate)	% Sequence Similarity VS116	UK	Am501
B burgdorferi ss			
(DK7)	98.8	98.7	98.9
(Lipitz)	99	98.9	99
B garinii			
(PBi)	99	98.9	99.1
(DK27)	99.1	99	99.2
B afzelii			
(DK1)	99.2	99.1	99.3
(DK21)	99.2	99.2	99.4
B japonica			
(HO14)	95.5	95.4	95.7
B bissettii			
(DN127)	96.2	96.1	96.4
B andersoni			
(21308)	97.6	97.5	97.8
B lusitaniae			
(PotiB1)	99.1	99	99.3
(PotiB2)	99	99	99.2
(PotiB3)	99.1	99	99.3
(IR345)	99.1	99	99.3
(BR41)	99.1	99	99.3
B valaisiana			
(VS116)	100.0	99.7	99.8
(UK)		100.0	99.0
(Am501)			100.0

valaisiana isolates have been cultured from ticks and birds in Europe (15 from Switzerland, 10 from The Netherlands, 3 from Germany, 2 from Spain, and 1 from the United Kingdom).[8,14,16,32,55] In Asia, *B valaisiana* was isolated from Korea, China, and Japan.[22]

The geographic distribution of *Borrelia* species is not uniform, even in close neighborhoods. In Western Europe and in Switzerland, however, *B garinii* is more frequently isolated, followed by *B afzelii, B burgdorferi* ss, and *B valaisiana,* respectively.[45,48] In Ireland, *B valaisiana* is described as the most prevalent genospecies, followed by *B garinii, B burgdorferi* ss, and *B afzelii.*[50,51] *B lusitaniae,* first isolated from *I ricinus* in Portugal, was subsequently isolated from ticks in other European countries.[36,56]

Vectors-Host Relationships of *B Valaisiana*

The principal vectors of *B valaisiana* are ticks of the *I ricinus* complex, that include *I ricinus* in Europe and *I persulcatus* in Asian Russia, China, and Japan.[8,14,16] *B valaisiana,* however, has also been detected in *Ixodes columnae* and *Ixodes granulatus* ticks in China and in *I nipponensis* ticks in Korea.[22,57] Therefore, the species

Table 7. List of accession numbers of B valaisiana fla *gene sequences available on GenBank DataBase.*

Isolates of *B valaisiana*	*n°* Accession	References
VS116	D82854	(41)
AM501	D82855	(41)
5MT	AB014677	(22)
10MT	AB014678	(22)

seem not to be strictly vector-specific.

Birds are assumed to be the only reservoir hosts for *B valaisiana* in Europe,[55] since, currently, no *B valaisiana* isolates have ever been cultured from any small or large mammals—including in Ireland where this genospecies is abundant in the tick population.[50] Therefore, this species may not survive or induce only short-term infections in small mammals. A complement system may play a key role for specific killing of some *Borrelia* genospecies among the various potential reservoir hosts.[58] *B valaisiana* seems particularly well adapted to birds.[49,55] It circulates between its avian reservoirs and ticks in natural foci, as *B garinii* does.[55] There is increasing evidence that the animal species do not transmit all *B burgdorferi* genospecies to ticks with equal efficiency.[49,55,59-62]

Mixed infections of multiple *B burgdorferi* sl species have been found in ticks, reservoir hosts, and in patients with Lyme borreliosis. *B valaisiana* transmission was often associated to *B garinii* transmission,[49,50,55] although mixed infection with *B valaisiana* and *B afzelii* also occurred.[44,46,54] *B garinii* and *B valaisiana* appear to have similar geographical distribution, as *B garinii* was predominant in most regions where *B valaisiana* was detected (England, Ireland, Germany, Switzerland, and Russia).[8,15,34,47,49] Moreover, in Ireland, where *B valaisiana* was described as a predominant genospecies, *B garinii* was also frequently detected.[50] These results point

Table 8. List of accession numbers of ospA *gene sequences available on GenBank DataBase.*

Isolates of *B valaisiana*	*n°* Accession	*n°* Accession of Partial Sequence (819 bp)	References
M53	AF095947		GenBank
M7	AF095943		GenBank
UK	Y10841		GenBank
AR-2	AF095942		GenBank
VS116	Y10840	AB016979	(22)
NE231	AJ249467		GenBank
5MT		AB016977	(22)
10MT		AB016978	(22)
Am501		AB016976	(22)

Table 9. Sequence identity matrix of partial sequences (819 bp) of **ospA** *genes of* **B valaisiana**[22] *(used with permission).*

Isolates	5MT	10MT	VS116	Am501
5MT	100	99.6	87.4	93.3
10MT	—	100	87.0	92.9
VS116	—	—	100	90.8
Am501	—	—	—	100

to possible similar ecosystems existing for *B valaisiana* and *B garinii*.[55]

PATHOGENICITY OF *B VALAISIANA* TO HUMANS

After the bite of an infected tick, the spirochetes undergo a hematogenous dissemination and can be found in many of the major organ systems. The first stage and hallmark of Lyme borreliosis is a distinctive skin rash, erythema migrans (EM), that frequently appears at the site of the tick bite.[63] It is not known if the different *Borrelia* genospecies induce this EM with the same frequency. Days to months after the tick bite, the disease may develop into its secondary and tertiary stages. In some patients, chronic diseases may develop that may affect the skin, such as Acrodermatitis Chronica Atrophicans (ACA), a clinical manifestation primarily observed in Europe. It may affect joints with arthritis, which is more common in the United States.[63] In Europe, neurological symptoms appear in 30% of untreated patients and musculoskeletal symptoms appear in 20% of patients.[64] These observations suggest that clinical outcome may depend on infection by strains of different genospecies. Neurological symptoms are thought to be mainly caused by *B garinii*, whereas *B afzelii* is often associated with ACA, and *B burgdorferi* ss with arthritis.[13,65-71]

Currently, only *B burgdorferi* ss, *B garinii,* and *B afzelii* have been frequently cultured from patients with Lyme borreliosis. However, *B valaisiana* has been detected by PCR in skin biopsies of two EM patients, and mixed infections including *B valaisiana* and another *Borrelia* species, were identified in both EM and ACA.[72] Moreover, immunoblots, with *B burgdorferi* ss, *B garinii, B afzelii*, and *B valaisiana* as antigens, revealed that 4 patients of 96 had a preferential reactivity to *B valaisiana*. Three of these patients had neurological symptoms (facial palsy) and the fourth patient had arthritis. These preliminary results provided some additional clues of the potential pathogenicity of *B valaisiana* in humans.[73] A recent study also alluded to the pathogenic potential of *B valaisiana.* This species has been amplified in synovial fluids from a patient suffering from Lyme arthritis. However, the presence of other *Borrelia* genospecies in this synovial fluid is not excluded.[21] Pathogenicity of *B valaisiana* is still questionable.

IMPLICATION FOR VACCINE DEVELOPMENT

The efficacy of the OspA-based vaccine in Europe may be considerably lower than in the United States, given the substantial variations displayed on OspA proteins of *B burgdorferi* sl. Immunization with recombinant OspA derived from one genospecies fails to protect against the others.[74] In Europe, the heterogeneous population of *Borrelia,* presenting in the natural focus, makes the development of a vaccine particularly difficult, especially against *B garinii* and *B valaisiana* that present heterogeneous OspA profiles.

DESCRIPTION OF B VALAISIANA

B valaisiana nov sp (va, lai, si, a' na), is in reference to the isolation canton—Valais—in Switzerland. Morphology is as described previously for the genus,[75] and cultural properties as described for *Borrelia*.[76] The rRNA gene restriction patterns after digestion by *Eco*RV contain three fragments (6.9, 3.2, and 1.4 kb) and after digestion by *Hin*dIII contain four fragments (2.1, 1.2, 0.8, and 0.6 kb).[16]

Most of the strains react in immunoblots with monoclonal antibody A116k directed to OspA,[21] p31c, or both.[22] No reactivity occurs with H3TS, I17.3, and D6, three species specific monoclonal antibodies to *B burgdorferi* ss, *B afzelii,* and *B garinii,* respectively. *B valaisiana* has been isolated from the ticks *I ricinus*, *I persulcatus*, *I columnae*, *I granulatus,* and *I nipponensis* in Europe and Asia, and from birds (Turdus merula) in Europe. The type of strain is strain VS116.

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Enhanced Macrophage Apoptosis in Patients With Lyme Disease

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ABSTRACT

The diagnosis of Lyme disease is commonly based on the patient's clinical presentation and associated serological response to *Borrelia burgdorferi* (Bb). However, there are some patients who do not show the diagnostic serological response to Bb. We investigated the possibility that macrophage dysfunction may be implicated in some instances of seronegative Lyme disease. Interaction between macrophages and microorganisms plays an important role in priming stimulation of lymphocytes for the production of antibodies. In the present study, to identify Lyme disease patients we used a sensitive and specific polymerase chain reaction (PCR) to detect Bb DNA coding for outer surface protein A (OspA 31 kda protein), which is a plasmid-located gene. We detected Bb targeting the DNA in macrophages and lymphocytes of seronegative Lyme disease patients using a nested PCR assay. In addition, an increased number of macrophages in seronegative patients were programmed to undergo apoptosis in vitro. Approximately 52% of the macrophages underwent apoptosis in OspA PCR positive patients (8 seronegative and 2 weak-seropositive), in contrast to 9% and 11% apoptotic macrophages in OspA PCR negative/strong-seropositive patients and healthy donors (as normal controls), respectively. We conclude that enhanced apoptosis, a reflection of altered macrophage programming of apoptosis may be considered an additional feature of seronegative Lyme disease patients.

Key words: lyme disease, *Borrelia burgdorferi,* outer surface protein A

INTRODUCTION

Lyme disease caused by the spirochete Bb is the most common tick borne infection in the United States. The initial stage of the disease is characterized by erythema migrans followed by an inflammatory process in the joints, nervous system, and heart.[1] Clinical diagnosis is based on the presence of the typical clinical signs and symptoms and serological evidence of Bb infection. There is a small subset of patients whose signs and symptoms are highly suggestive of Lyme disease, but lack serological evidence to confirm the diagnosis.[2,3,4] In such "seronegative" patients, the humoral immune response appears to be blunted or aborted. Some have suggested that early and/or incomplete antibiotic treatment may be the cause.[5] Other possibilities include immune complexes that bind all of the free antibodies.[6] It is also possible that these individuals have undefined immunological defects resulting in a failure to mount an antibody response.[7,8]

In bacterial infections the induction of apoptosis has been associated with suppression of immune response.[9] Macrophages, are highly active phagocytic cells, present in both blood and tissue. Antigen-presenting cells, such as macrophages, play a crucial role in the processing and presentation of antigens.[10-12] The presence of Bb DNA in macrophages and the role of macrophages in the killing of Bb have been described.[13-15] Incomplete clearance of Bb by phagocytic cells results in persistence of Bb in chronic and recurrent Lyme disease.[14] We investigated whether human macrophages could be persistently infected with Bb and to see if there was any difference in the rate of apoptosis of Lyme disease patients.

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MATERIALS AND METHODS

Patients

Patients undergoing venipuncture for diagnosis of Lyme disease at the Lyme Disease Center of Long Island Jewish Medical Center were asked to submit 25 mL of whole blood and/or 1 mL cerebrospinal fluid (CSF) by lumbar puncture. Healthy employees of the Long Island Jewish Medical Center consented to have their blood drawn as well. There were three groups of patients: 1) Lyme disease patients with a history of exposure, clinical signs and symptoms, and confirmatory serology (positive ELISA and Western blot); 2) seronegative patients with signs and symptoms and exposure to Bb without confirmatory serologies; and 3) normal controls from healthy donors without Lyme disease exposure. Seronegative patients were referred to the Lyme disease center because of their exposure history and symptoms suggestive of Lyme disease. The samples were blinded and then sent to the laboratory. To ensure the specificity of the PCR test we also included samples from non-Lyme disease patients (5 degenerative arthritis, 1 multiple sclerosis, and 1 cerebrovascular accident).

Serological Tests

Tests of Bb-specific immunoglobulin G (IgG) and IgM antibodies were performed using Bb IgG/IgM Enzyme-Linked Immunosorbent Assay (ELISA, Wampole Laboratories, Cranbury, NJ) and Bb IgG/IgM Marblot Strip Test System (Western blot kit, MarDx Diagnostic Inc., Carlsbad, CA) according to the manufacturer's instructions. All the patient groups, including the 7 with other diseases were tested.

Cell Isolation

Macrophages and lymphocytes were isolated and tested for Bb. In brief, 25 mL of whole blood from Lyme disease patients and healthy donors were collected in heparinized tubes. Mononuclear cells were isolated by centrifuging whole blood over Lymphoprep (GIBCO Life Technologies, Gaithersburg, MD); the mononuclear cell layers were cultured in 25 cm^2 flasks and on glass chamber slides for 48 to 72 hours[16] (RPMI 1640 medium with 10% fetal bovine serum, 5% CO_2 incubation at 37°C). Nonadherent cells (lymphocytes) were separated and washed. Nested PCR was performed on both adherent and nonadherent cells growing in the flask. For preparation of slides, nonadherent cells were removed from the chamber slides and plated on clean microscope slides. Adherent cells were washed 3 times with PBS then air-dried. Both fractions were fixed in 100% acetone at -20°C for 3 minutes followed by staining.

PCR

Ten μL TE buffer was added to each of the pellets from 1/5 the macrophage fraction (released by scraping) and 1/10 the lymphocyte fraction, then boiled for 30 minutes. One mL CSF was obtained from patients by lumbar puncture. CSF was centrifuged at 16,000 × g for 30 minutes, 10 μL TE buffer was added to the pellets then boiled for 30 minutes. $MgCl_2$ was added to saturate the TE buffer. The nested PCR was modified from a previously published procedure.[17] For OspA PCR, the first reaction mixture of 50 μl contained 10 μL cell lysate, 1 × PCR buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.25 μmol/L of each external primer and 1.5 U of Taq DNA polymerase. The amplification condition was denaturing 1.5 minutes at 94°C, annealing 2 minutes at 45°C, and elongation 2 minutes at 72°C, for 30 cycles. Ten μL product of first reaction was added to a new PCR mixture containing 1 x PCR buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.25 μmol/L of each internal primer and 1.5 U of Taq DNA polymerase. Amplification conditions were changed to 35 cycles and annealing temperature to 55°C. PCR amplification was performed in a Perkin-Elmer DNA thermal cycler. The sequences of OspA PCR primers used and expected size of PCR products were external primers, 5′-GGGAATAGGTCTAATATTAGCC-3′ (forward) and 5′-CACTAATTGTTAAAGTGGAAGT-GCC-3′ (reverse), 622 bp; and internal primers, 5′-GCAAAATGTTAGCAGCCTTGACG-3′ (forward) and 5′-CTGTGTATTCA-AGTCTGGTTCC-3′ (reverse), 392 bp. PCR products were analyzed on a 1.5% agarose gel. Ten fg of Bb B31 DNA served as a positive control. To ensure that the DNA was amplifiable, 1 μL of cell lysate was used to amplify exon 17 of the human amyloid precursor protein (APP) gene.[18] The APP primers were purchased from Research Genetics, Inc. (Huntsville, AL). For APP, PCR was performed with 35 cycles of 1 minute at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C. The PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. A 50 to -2000 bp DNA ladder (Bio-Rad, Hercules, CA) was loaded as a size marker. The PCR hoods were UV sterilized for at least 30 minutes prior to use. Each step in the nested PCR was done in a different room using a different hood. Both negative and positive controls were used in each assay. The positive control was never added in the same room in which the PCR was set up.

Apoptosis Assay

Evaluation of apoptosis in macrophages was performed using the In Situ Cell Death Detection Kit (Boehringer Mannheim Corporation, Indianapolis, IN). Briefly, the slides prepared as described above were incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium

Table 1. Clinical profile, serology and OspA PCR products of Lyme disease patients in this study.

Patient No.	Age	Sex	Duration	Signs and Symptoms	Serology G	Serology M	Serology E	OspA PCR Pre	OspA PCR Post	Apoptosis (%±ER)	Improvement
1	24	F	1 year	hip pain and fatigue	-	-	-	+	-	46±10	yes
2	47	F	1 year	memory change, fatigue, and radiculopathy	-	-	-	+	-	33±2	yes
3	56	F	6 months	knee pain, effusion, and headache	-	-	-	+	-	55±5	yes
4	56	M	2 months	fever and EM rash	-	-	-	+	-	56±7	yes
5	43	M	3 weeks	EM rash, GBS, and Bell's palsy	-	-	-	+	NT	67±11	yes
6	30	M	3 years	headache, fatigue, knee effusion, short-term memory loss, and parathesias	-	-	-		NT	63±15	yes
7	37	F	1 year	joint pain, fatigue, flu-like, knee pain, and memory changes	-	-	-	+	-	61±13	yes
8	20	F	1 month	seizure, memory changes, and bilat knee fluid	-	-	-	+	NT	39±4	yes
9	44	F	4 months	flu-like, EM rash, and headache	-	+	+	+	-	48±9	yes
10	54	F	5 weeks	flu-like, headache, and disseminated EM	+	+	+	+	-	58±13	yes
11	45	M	6 months	Bell's palsy	+	+	+	-	NT	5±0.5	yes
12	37	M	6 years	joint pain and effusion	+	+	+	-	NT	7±0.0	yes
13	70	M	3 months	EM rash, flu-like, and headache	+	+	+	-	NT	11±5	yes
14	6	M	2 weeks	EM rash, flu-like, fever, and joint pain	+	+	+	-	NT	10±5	yes
15	74	M	8 weeks	arthralgias, fatigue, dementia, and ataxia	+	+	+	-	NT	4±0.1	yes
16	58	F	7 years	arrythmias, memory changes, and encephlopathy	+	+	+	-	NT	14±3	yes
17	53	F	4 months	EM rash, fatigue, memory loss, and encephalopathy	+	+	+	-	NT	9±5	yes

G, IgG; M, IgM; E, ELISA. ER, standard error; EM, erythema migrans; NT, not tested; Pre: Before antibiotic treatment; Post: After antibiotic treatment.

citrate) for 2 minutes at 4°C and then rinsed twice with PBS. Fifty μL of a terminal dexynucleotidyl transferace-mediated (TdT) dUTP nick end labeling (TUNEL) reaction mixture was then added onto the slides. The slides were incubated in a humidified chamber for 60 minutes at 37°C. After a complete rinse with PBS, the slides were exposed to 50 μL converter-AP for 30 minutes. After 3 rinses a substrate solution (50-100 μL) was added further incubated for 10 minutes at room temperature. After a complete wash, they were air-dried and mounted.

The slides were viewed under a light microscope and photographed at 400× using Kodak Gold 100 film. Apoptosis was indicated by the appearance of brightly-labeled nuclei and apoptotic bodies. Quantification of apoptosis in 5 different fields on each slide was carried out independently by two investigators after the slides were blinded during the cell count. We also used a mouse antihuman macrophage/CD11b (Boehringer Mannheim Biochemica, Indianapolis, IN) and monoclonal mouse antihuman macrophage IgM clone HAM 56 antibody[19] (Enzo Diagnostics, Farmingdale, NY) to identify mononuclear cell populations on both adherent and non-adherent cells.

Statistical Analysis

The values for apoptotic macrophages were expressed as means and standard errors. To compare the values amongst different groups, a Student-Newman-Keuls multiple comparisons test was used.

RESULTS

Included in this study were 17 patients with Lyme disease and 12 controls (5 healthy patients and 7 without - Lyme disease as described in methods). The clinical characteristics of the patients with Lyme are listed in **Table 1.** Among the 17 Lyme patients, 8 were males and 9 were females, ranging in age from 6 to 74 (mean, 44.5 years). By serological examination using ELISA and Western blot assays, 8 patients were seronegative and 9 patients were seropositive for antibodies to Bb as summarized in **Table 1**. Of the 9 seropositive patients, 6 had predominantly arthritic complaints, and 3 had neurological. Of the 8 seronegative patients, 4 patients had predominantly arthritic complaints and 4 neurological. All of the patients with Lyme disease were subsequently treated with either doxycycline PO or ceftriaxone IV for a period of 30 to 40 days. The 5 healthy subjects and 7 non-Lyme disease patients, all tested negative by Lyme ELISA and Western blot assays.

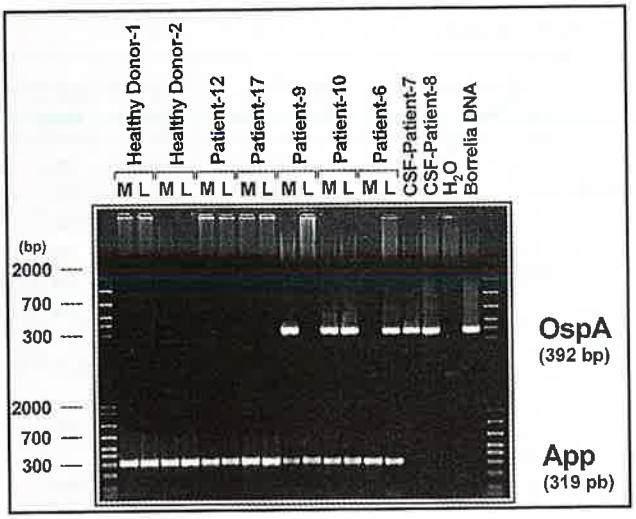

Figure 1. Qualitative PCR assay for Borrelia burgdorferi *in macrophages and lymphocytes from Lyme disease patients. Assay of Bb was carried out by nested PCR described in "Materials and Methods" in a representative sample set from each group of seronegative/OspA PCR positive (patients 6, 7 and 8), weakly-seropositive/OspA PCR positive (6 and 10), strongly-seropositive/OspA PCR negative patients (12 and 17), and healthy donors. Ten fg of Bb B31 DNA was used as a PCR positive control, and H_2O as a PCR negative control. M, macrophage; L, lymphocyte.*

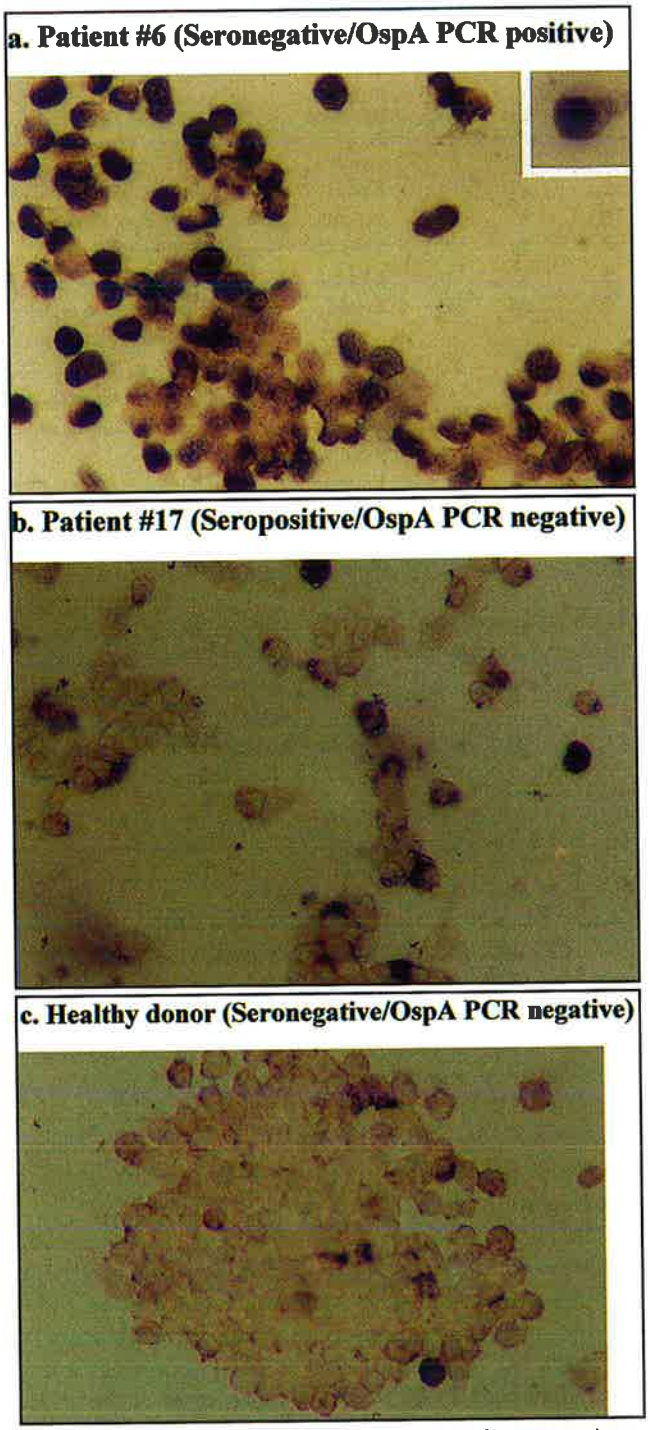

Figure 2A. Apoptotic macrophages in Lyme disease patients. Representative morphological changes taken under 400 x magnification without phase contrast from each group of seronegative/OspA PCR positive patients (patient 6), seropositive/OspA PCR negative patients (patient 17), and a healthy donor after staining of macrophages using TUNEL assay as described in "Materials and Methods." A photograph of a typical apoptotic macrophage was taken under 1,000 x magnification (right corner of top picture).

Detection of Bb by Nested PCR

The OspA PCR products were detected either in the macrophage/lymphocyte fraction or in both fractions of the cell lysates of 8 seronegative and 2 weakly seropositive patients **(Table 1).** Seven strong-seropositive patients, all healthy subjects and non-Lyme disease patients showed OspA PCR negative results in their cell lysates. **Figure 1** shows a representative set of OspA PCR products in the patients. Two healthy donors (as controls), two seropositive patients (patients 12 and 17), three seronegative patients (patients 6, 7, and 8), and two weak-seropositive patients (patients 9 and 10) were included. A marked amount of OspA PCR products (392 bp) were observed in either macrophages (M) or lymphocytes (L) in patients 6 and 9, and in CSF of patients 7 and 8. In patient 10, OspA PCR products were seen in both macrophages and lymphocytes. A H_2O sample, as a PCR negative control, and a Bb DNA (10 fg) sample, as a positive control, were included in the gel. The macrophages and lymphocytes of non-Lyme disease patients tested by OspA PCR had consistently negative results. Post-treatment PCR was done on patients 1, 2, 3, 4, 7, 9, and 10. One month after the initial PCR patient 1 remained positive. After two months of treatment this patient became PCR negative. Patient 10 was PCR positive for at least 8 months after treatment, showing a negative PCR after the tenth month of treatment. Patients 2, 3, 4, 7,

and 9 were PCR negative after one course of treatment. None of the seronegative patients converted to seropositive after treatment. The PCR product (319 bp) of the human APP gene was amplified on every specimen to ensure that every sample contained amplifiable DNA. PCR positive and PCR negative samples were confirmed by Southern blot using two different probes, oligo (within the PCR product sequence) and the full-length of the PCR product, to validate the specificity (data not shown). The majority (> 90%) of adherent cells were confirmed to be macrophages (positive staining with mouse antihuman macrophage antibodies), whereas less than 10% cells in the nonadherent fraction stained positive (data not shown).

Apoptosis by TUNEL Assay

DNA during apoptosis yields double-stranded breaks into low molecular weight DNA fragments. These DNA fragments can be identified by labeling free 3-OH termini with nucleotides in a TdT-mediated reaction. Using an In Situ Cell Death Detection Kit, there was a marked difference in staining density between apoptotic macrophages and non-apoptotic macrophages (**Figure 2A**). The macrophages from the OspA PCR positive patients exhibited a significant increase of apoptosis when compared with the macrophages of OspA PCR negative patients and healthy subjects (**Table 1**). Post-treatment apoptosis assays were done on patients 2, 3, and 10. Two had an increase in apoptosis while the third showed no change. The representative morphological changes taken under 400× magnification without phase contrast are shown in **Figure 2A.** Although typical apoptotic morphological changes, cytoplasmic contraction and chromatin condensation, were observed in all samples; apoptosis induction was seen in approximately 52 ± 5% macrophages of OspA PCR positive patients, whereas OspA PCR negative patients and healthy subjects showed only about 9 ± 1% and 11 ± 2% of the apoptotic macrophages, respectively (**Figure 2B**). The difference in means and medians of apoptosis percentage were analyzed by a Student-Newman-Keuls Multiple Comparisons Test (two-tails). Statistical analyses illustrated a significantly different percentage of apoptosis induction between the group of OspA PCR positive patients and the group of OspA PCR negative patients, and between the group of OspA PCR positive patients and the group of healthy donors ($P < 0.001$ for both comparisons). In contrast, there was no difference between the group of OspA PCR negative patients and the group of healthy donors ($P > 0.05$).

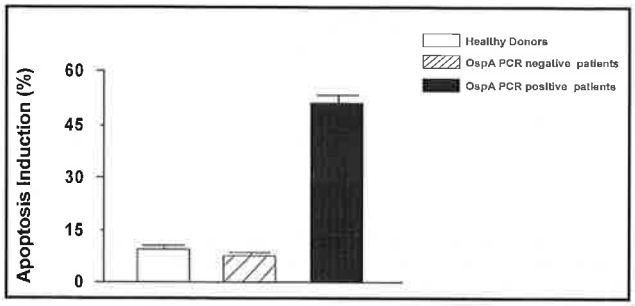

Figure 2B. A comparison of percentage of apoptotic macrophages between each group. Bar, standard error.

DISCUSSION

It has been known that macrophages play an important role in the host immune system, inflammatory responses, phagocytosis, and antigen presentation.[12,20] In some infectious diseases microorganisms can initiate infection, multiply intracellularly and escape from the host immune response.[21] Moreover, Bb has been shown to attack macrophages and lymphocytes.[13,22] Consistent with these observations, our studies demonstrate that OspA DNA is present in the macrophages of seronegative and weak-seropositive Lyme disease patients rather than strong-seropositive Lyme disease patients as shown in **Table 1** and **Figure 1.**

Apoptosis, programmed cell death, is a mechanism to remove dead cells from normal tissues to maintain homeostasis. A condensed nucleus and cytoplasm, blebbing of the plasma membrane and DNA fragmentation characterize cells undergoing apoptosis.[23] We previously reported that drugs such as ethanol and morphine compromise immune function through the induction of apoptosis in macrophages.[16,24] Also, other researchers demonstrated that apoptosis is induced in macrophages by a variety of microorganisms.[19,25,26] Moreover, animal studies show that inflammatory responses to polymicrobial sepsis may suppress immune cells by inducing apoptosis.[27] In the present study, we observed that approximately 50% of macrophages obtained from OspA PCR positive Lyme disease patients underwent apoptosis compared to less than 10% apoptosis induction in OspA PCR negative patients and healthy subjects.

Taken together, the data in this study provide the first evidence for high frequencies of apoptotic macrophages in seronegative/OspA PCR positive Lyme disease patients and suggest that Bb-induced apoptosis in seronegative Lyme disease patients may be important for initiation of infection and escape from host immune responses. The reason patients responded to Bb differently is not clear. It may be genetic differences; however, this phenomenon requires further probing. An insight into the relationship between apoptosis and serology in Lyme disease patients may be of clinical importance to support the diagnosis as well as for treatment follow-up.

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INFORMATION FOR CONTRIBUTORS

Journal of Spirochetal and Tick-borne Diseases

Dedicated to science and art in spirochetal and tick-borne diseases

INFORMATION FOR AUTHORS AND EDITORIAL POLICY

The following guidelines are in accordance with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" and the International Committee of Medical Journal Editors (the "Vancouver Group") statement, agreed at the January 1993 Meeting.

The *Journal of Spirochetal and Tick-borne Diseases* publishes quarterly reviews and original work studies about any aspect of spirochetal and tick-borne diseases. The primary purpose is to broaden our understanding of spirochetal and tick-borne diseases. Special focus is given to Lyme borreliosis (also known as Lyme disease), as the most prevalent spirochetal and tick-borne disease. The clinical topics may involve all medical disciplines, nursing, and pharmacy, as well as the social, ethical, and biological features of spirochetal and tick-borne diseases.

The Journal is composed of two major sections. One section is devoted to the review of a specific topic that is established by the Associate Editors, and a special guest editor is invited to coordinate the development of up to six manuscripts relating to the specific topic. The second section of original works is composed of unsolicited manuscripts that are subsequently reviewed by the Review Board, as well as external reviewers, depending on the potential for conflict of interest within the editorial panel and the potential interest by the readership.

Expeditious review of all manuscripts is carried out with a projected response time of not more than 6 weeks. Rejected manuscripts usually are returned to authors within 6 weeks. Decisions about potentially acceptable manuscripts may take somewhat longer.

The Journal will publish material defined within the categories described in the following.

Reviews

Each issue includes a series of state-of-the-art articles on a topic related to spirochetal and tick-borne diseases. The articles represent invited presentation by authorities in the field on topics related to spirochetal and tick-borne diseases, with an emphasis on Lyme borreliosis.

Each manuscript should present a comprehensive state-of-the-art analysis and should be accompanied by an abstract of 300 words or less summarizing major points.

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Original articles of 5000 words or less may be submitted to the editorial office Each article should be accompanied by an abstract of 300 words or less describing the findings of the original research. All articles will be peer reviewed within a 3-week period with subsequent notification to the authors within 5 weeks of submission. Submitted articles may relate to any area of spirochetal and tick-borne diseases.

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Specific clinical case reports describing a unique approach to Lyme disease and other related disorders in the area of diagnosis or treatment may be submitted for review. An abstract of 250 words or less should accompany the text.

Correspondence

Letters to the Editor in the form of correspondence related to material published in the Journal or some aspect of Lyme borreliosis and other spirochetal and tick-borne diseases may be submitted. Such letters, if related to work previously published in the Journal, will be referred to the author of the original work for a response. Letters to the Editor should be submitted in duplicate, typewritten and double-spaced, not exceeding 400 words of text and a maximum of five references. Letters should have no more than three authors, and should be signed by all of the authors. Please include a word count. Receipt of letters is not acknowledged, but correspondents will be notified when a decision regarding publication is made.

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All material should be typed and double-spaced. Standard sequence of methods and materials, results, and discussion should be employed with tables and figures numbered in the order in which they are cited in the text.

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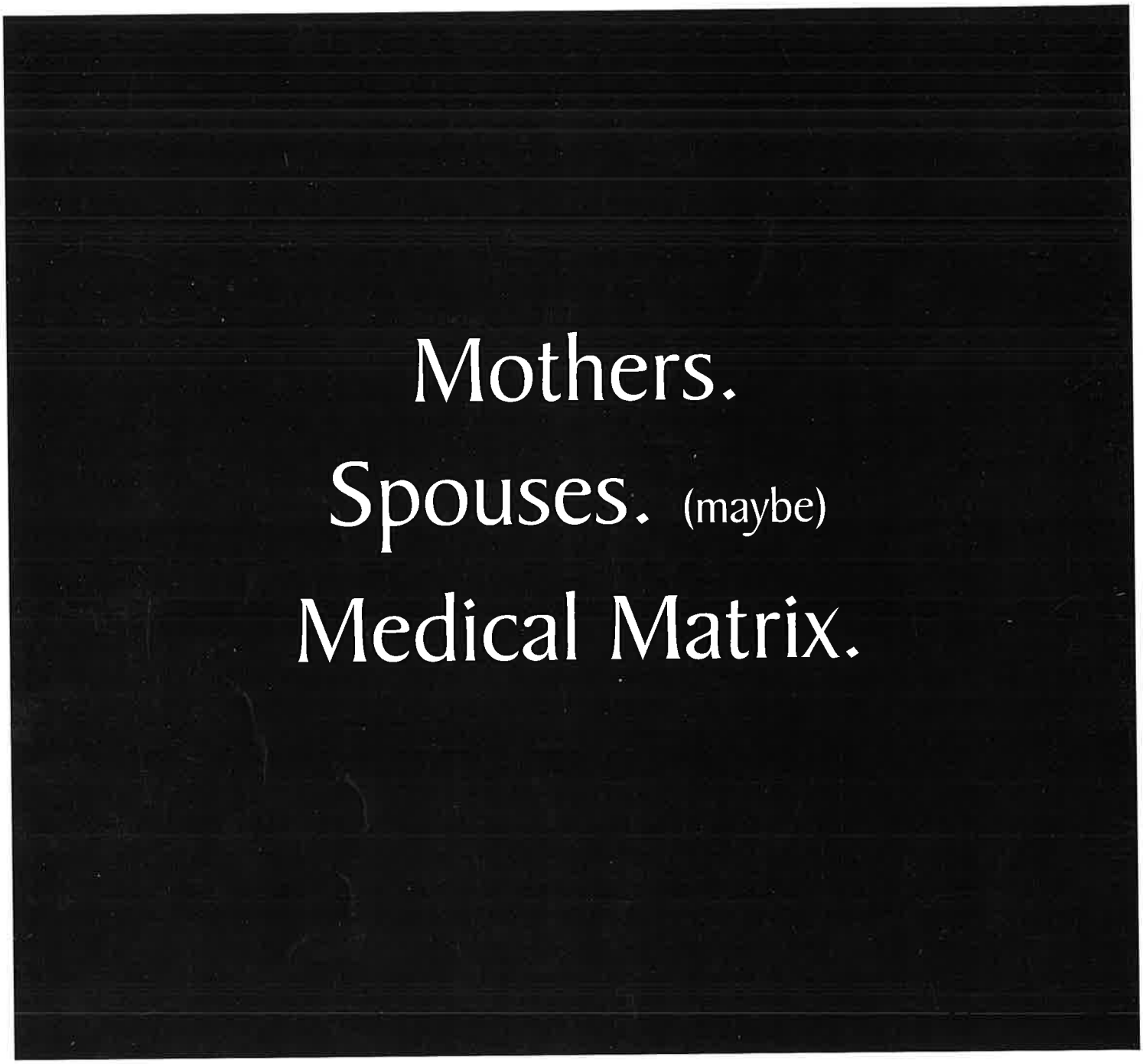

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