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MOLECULAR AND MICROSCOPICAL EVIDENCE OF *Ehrlichia spp.* AND *Borrelia burgdorferi sensu lato* IN PATIENTS, ANIMALS AND TICKS IN THE CZECH REPUBLIC

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SUMMARY

We report moderately severe cases of human ehrlichiosis and a lethal one caused by human granulocytic *Ehrlichia,* the HGE agent, closely related to *Ehrlichia phagocytophila* and *Ehrlichia equi.* Their vector is the *Ixodes ricinus* tick, which also transmits *Borrelia burgorferi sensu lato* in central, west and east regions of the Czech Republic. The diagnosis was established by PCR with sequence analysis of the genes encoding 16S rRNA of *Ehrlichia* and with reverse hybridization by using enzyme linked immunosorbent assay with different covalently coupled probes to the activated plate.Ten out of 47 patients and 10 huntsmen were PCR positive and 7 of them seroconverted to the HGE. Coinfection of *Ehrlichia phagocytophila* with *Borrelia burgdorferi sensu lato* was detected in 3 patients. *Ehrlichia* spp.. the HGE agent, was isolated and propagated only from one patient in the HL-60 promyelocytic cell line. The maintenance of *Ehrlichia* in culture and in patients was assayed also by immunocytological staining and electron microscopy.

Sequence or hybridization analysis of PCR results in different wild mammals and birds showed significant sources of *Ehrlichia fagocytophila* in nature. Three variants of *E. phagocytophila* in wild roe deer and boars, as well as for the first

time in birds, have been described. Cultures from the blood of horses, and from the spleen and kidney specimens of roes and boars, PCR positive for *Ehrlichia* spp., displayed a disappearing level of the pathogen or contamination with other bacteria.

KEY WORDS: *Ehrlichia phagocytophila*, HGE agent, *Ehrlichia equi*, polymerase chain reaction (PCR), sequence analysis, ELISA plate reverse hybridisation, HL-60 cell line, electron microscopy, infection of humans, animals, coinfection, *Borrelia burgdorferi sensu lato*

INTRODUCTION

Lyme borreliosis, ehrlichiosis and bartonellosis are zoonotic vector-borne emerging infectious diseases. Serologic evidence of HGE in Danish patients (Lebech *et al.*, 1998) and molecular analyses (Engvall *et al.*, 1996) indicate that human granulocytic ehrlichia (HGE) in the *Ehrlichia phagocytophila* genogroup (i.e. *E. phagocytophila* infecting ruminants, *E. equi* infecting horses) are also widespread in Europe. *E. phagocytophila* may also cause infections of cats, cattle and dogs (Engvall *et al.*, 1996). Together with a human monocytic ehrlichia (HME), *Ehrlichia chaffeensis* (Anderson *et al.*, 1991), HGE has been recognised as the cause of coinfection with *Borrelia burgdorferi sensu lato* in ticks (Schouls *et al.*, 1999) and in patients with Lyme borreliosis (Christova and Dumler, 1999). The central nervous system (CNS), heart, kidney, liver and synovium of joints are common target organs for *B. burgdorferi sensu lato* and also for *Ehrlichia* spp. (Walker and Dumler, 1997). Correlation between neuroborreliosis and infection with *B. garinii* OspA serotype 4 strains was described by Marconi *et al.*, (1999). *E. canis* and HME were also identified in acute or chronic infection with neurologic symptoms (Walker and Dumler, 1997). *E. ewingi* is considered to cause polyarthritis (Breitschwerdt *et al.*, 1998) in dogs similar to that caused by *Borrelia* sp. Clinical manifestations caused by *E.equi* and HGE are mentioned to be milder (Bakken *et al.*, 1996) than HME symptoms and often nonspecific in acute infection. Clinical diagnosis of HGE infection or coinfection with Borrelia is difficult. Diagnosis of both infections often relies heavily on laboratory tests. Culturing of HGE (Goodman *et al.*, 1996, Heimer *et al.*, 1997) and *Borrelia* sp. has low sensitivity. Microscopic examination requires special expertise. The specificity and sensitivity of PCR analyses (Engvall *et al.*, 1996, Liz *et al.*, 2000, Harrus *et al.*, 1998) for the detection of HGE and for identification of *B. burgdorferi sensu lato* (Vasiliu *et al.*, 1998) probably exceed those of other methods.

In the present study we describe the results of microscopic methods and a combination of PCR technology using 16S rRNA or OspA sequence analyses applied to recognise infection or coinfection with *Ehrlichia* species and *Borrelia* species in humans, animals and ticks.

MATERIAL AND METHODS

Patient selection

A total of 47 patients with different manifestations of Lyme borreliosis (LB) and 10 huntsmen after tick bite were ascertained in the period 2000 – 2001. Eleven patients with neuroborreliosis (Bannwarth's syndrome, and meningitis with facial palsy and detected pleocytosis or intrathecal antibodies), 23 with erythema migrans (EM) and 10 patients with fever myalgias, gastrointestinal symptoms and positive serology for LB, from central, west, east endemic regions of the Czech Republic (CZ) were selected.

Medical records of 10 out of 47 patients and of 10 huntsmen who had HGE diagnosed in our labo-

ratory were reviewed. These patients had an anamnesis of tick bite, and a) detectable anti-HGE agent antibodies, b) a positive result of PCR analysis of the blood or CSF samples, and /or c) abnormal laboratory values in the blood count (leukopenia, thrombocytopenia, lymphocytosis, etc.), and/or presence of morulae in peripheral blood neutrophils.
Twenty-one blood samples obtained from blood donors were used as a control.

Animals
Records of 236 animals included 95 game animals or their cadavers obtained from the State Veterinary Institute (harts *Cervus europeus)*, roes (*Capreolus capreolus)*, foxes *(Vulpes vulpes)*, boar *(Sus scrofa),* hares (*Lepus europaeus)*, moufflon (*Dama dama*) and 48 shot birds (*Phasianus colchicus, Anas platyrhinchus, Columba palumbus, Larus ridibundus, Corvus frugilegus, Garrulus glandarius, Phalacrocorax carbo*, etc), 38 young horses imported from France, and the 55 small mice (*Apodemus flavicolis, Microtus arvalis, Clatrionomys glareolus*) from east CZ and from the horse-breeding farm were investigated. Samples of animal tissue, namely spleen, muscle or heart and kidney were refrigerated (-20°C) and processed later for PCR.
Ninety *Ixodes ricinus* ticks, collected by flagging in forested areas near a horse pasture and some removed from roe deer shot in eastern CZ, were also investigated. *Lipoptera cervi* nymphs and adults were removed from shot animals and sheep.

Cultivation
Acute–phase blood was collected from 4 of 10 tick bite patients as EDTA-or citrate-anticoagulated blood on days 5, 10, 16 after onset of illness (EM, fever and/or neurological symptoms). Periferal blood smears from these patients were examined in Giemsa-Wright staining.
The blood plasma was centrifuged at 2,500 rpm for 10 min and the buffy coat was washed in RPMI 1640 medium supplemented with 20% fetal bovine serum, pyruvate and glutamine, harvested and used for infection of HL-60 cells maintained in 12.5 cm^2 tissue culture flasks (1ml HL-60 cells at 10^6 cells/ml, 1 ml buffy coat washed in medium and 5 ml of RPMI 1640 medium with supplements) as described elsewhere (Heimer *et al.*, 1997). After 3 days, 2 ml of culture were removed from the initial culture to a new flask. The maintenance of HGE in cultures was assayed just as in animal and human tissue samples, by polymerase chain reaction (PCR) and light or electron microscopy observations using negative staining with 1% phosphotungstic acid (PTA) and Lowicryl K4M embedded semithic or ultrathic sections (Hulínska *et al.*, 1999).

Serologic tests
Lyme serology was performed using full-antigen enzyme-linked immunosorbent assay (ELISA) and Western blotting (Biowestern, Ltd). Serum samples from 47 patients and 10 huntsmen were screened for antibodies applying the IFA method, as desribed elsewhere (Mitchell *et al.*, 1996).
HL-60 promyelocyte cells infected with the HGE agent and HME in the ID8 cell line on slides (MRL diagnostics, Ltd, USA) were used. Anti-ehrlichial HGE IFA IgG and IgM antibody titers were estimated to be positive as recomended by the supplier. The positive control should endpoint (1+ fluorescence) at 1:8 i.e. 8-fold beyond the bottled dilution. Interpretation of the declared IgM serum endpoint titers of 1:20 and greater, and IgG serum endpoint titers ≥1:64 as presumptive evidence of recent or current HGE infection.

Nucleic acid extraction
Two different methods were used for isolation of borrelial and ehrlichial DNA from anticoagulated blood, CSF and tissue samples: alkaline lysis, and commercially available Invisorb Genomic DNA Kit III as recommended by the supplier (Invitek, Ltd, Germany).

Nucleic acid amplification (PCR)
We used four primer sets for the detection of DNA *Borrelia* spp. and *Ehrlichia* spp. DNA. One targeting *B. burgdorferi sensu lato* specific chromosomal gene segments (Marconi and Garon, 1992), one for a plasmid located OspA gene (Vasiliu *et al.*, 1998). Amplification of ehrlichial DNA was arranged using primers ECC, ECB (Harrus *et al.*, 1998) and nested inner HE3 primer and HE3-R primer (Breitschwerdt *et al.*, 1998) with specific probes for *E. equi, E. phagocytophila* and *E. canis*. Another primer, 593II, corresponding to the universal U1 region and reverse primer ER 2 (Engvall *et al.*, 1996) were used for direct sequenation of the DNA amplification product. PCRs were performed in a 50 µl volume containing 1 or 2 µl of template DNA, 10 pmol of each primer, 10 mM Tris-HCl buffer, 200 mM deoxynucleotide triphosphate, and 1.0- 1.5 U of Taq DNA polymerase. The number of cycles and cycling conditions in a PTC-200 (MJ Research) thermocycler were as had been described by previous authors. Positive and negative controls were in-

cluded in each run. Positive control was DNA from our culture, negative control was DNA isolated from blood donors. PCR 100 bp low marker (Sigma, cat. No. D7058) was used in analysis of the PCR products by electrophoresis in 1.5% agarose, stained with ethidium bromide. Distinct amplicon bands (products) were analysed by modified reverse hybridization (Schouls *et al.*, 1999) in enzyme-linked immunosorbent essay (ELISA) plates or by direct sequenation.

ELISA plate-hybridization used 5´amino-linked oligonucleotide probes as described by Harrus *et al.* (1999) and Schouls *et al.* (1999). Probes were covalently coupled to the activated ELISA DNA-Bind plate strips by adding of 100 µl/well at a concentration of 25 pmol/well in oligo binding buffer (50 mM Na2PO4, 1 mM EDTA) and incubated overnight. Plates were washed 3 x with PBS and blocked by adding 3% bovine serum albumin (BSA) in oligo buffer at 37°C and then decanted. Biotin-labeled PCR products were diluted with hybridization solution (5x SSC, 1% casein, 0.02% sodium dodecyl sulfate, 0.1% A-lauroylsarcosine), denatured for 10 min at 98°C and cooled in an ice- ethanol bath. Hybridization was performed for 1.5 h at 42°C in a Micro-hybridization Incubator (Robbins Scientific, model 2000). Wells were washed with 2x SSC, 0.1% SDS at 34°C and blocked with blocking solution for 30 min and subsequently incubated for 30 min at 42°C with streptavidin peroxidase (Boehringer Mannheim, GmbH) diluted 1:1000 in blocking solution. Wells were washed three times with PBS containing detergent (Tween 20) and then were incubated with substrate solution containing DAB (3,3´ diaminobenzidine tetrahydrochloride dihydrate, Fluka GmbH). The optical densities of the DNA-Bind wells were read at 405 nm in a 96-well plate reader Dynatech MR5000. Signals should be above 0.6 absorbance while the background should be well below 0.1.

Nucleotide sequencing

Positive products (395 bp) from nested PCR for the OspA gene of *B. burgdorferi sensu lato* and 619 bp products corresponding to the U_1 region of upstream primer 593II and the U5 region of downstream ERB2 primer (Engvall *et al.*, 1996) or the 390 bp product obtained with the inner primers, HE3-R and HE3 (Harrus *et al.*, 1999) derived from the 16S rRNA gene of *E. equi, E. canis, E. phagocytophila* were purified using the MultiScreen PCR kit (Milipore GmbH). PCR amplified DNA was prepared for sequencing on the MJ Research PTC-200 thermocycler (96°C for 20 s, 50°C for 20 s, 60°C for 4 min, 30 cycles). Direct sequencing of PCR products was done by the dideoxy chain termination procedure by using the CEQ 2000 XL automatic sequencer. The Sequencing kit (Beckman Coulter) uses unlabelled primer sets.

The following GenBank accession numbers were used to retrieve the corresponding 16S rRNA sequences: AF 136712 for *E. phagocytophila*, strain Frankonia II, AF 136714 for *E. phagocytophila* genogroup strain Baden, AF 482761 for *E. equi*, AF189153 for the HGE agent, M73226 for *E. canis*, M73222 for *E. chaffeensis.*

GenBank accession number AF 227319 for *B. garini*, OspA serotype 3, X85440 AF227317 for *B. garinii* serotype 5, AF227319 for serotype 4, U20360 for *B. burgdorferi sensu stricto*, L23137 for strain B19CT, and U20356 for *Borrelia afzelii.* The collected sequences were analysed with the BLASt and DNAStar package.

RESULTS

Ehrlichia phagocytophila and/or the *HGE* agent diagnosed by PCR in 10 out of 57 humans, in 28 out of 236 (11.8%) animals and *Borrelia burgdorferi sensu lato* diagnosed by PCR from 33 out of 236 (13.9%) animals, all from June 2000 through December 2001 in the central, west and eastern regions of CZ summarized in Graph 1. Ticks were not included because all were collected in the autumn. Infectious rates in game animals,or their cadavers, birds, horses, mice and ticks are shown in Table 1.

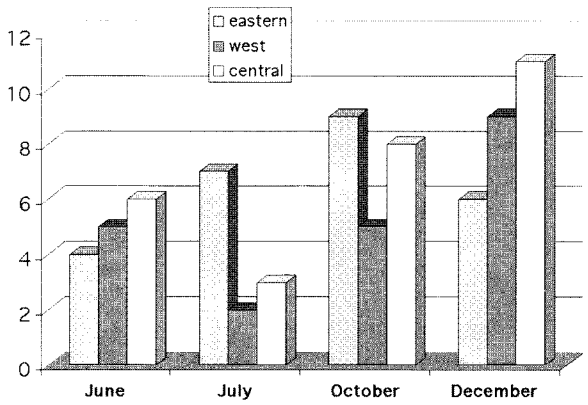
Medical records of 10 patients positive for *Ehrlichia spp.* out of 47 and 10 huntsmen included the following case reports:

No. 1. was a 67-year-old man. The patient had been treated for psoriatic arthritis with corticoids (Metotrexat 15 mg/ a week, Diclofenac 50 mg 2 x 1, Medrol 16 mg/ a day) before the tick bite. In January 2000 he had multiple erythema migrans (EM), he was treated with doxycycline (2g/day), EM disappeared in the course of treatment. The hospitalized patient had a 3-day fever up to 39.0°C and renal insufficiency. The patient died in hospital on May 22 with a picture of multiorgan failure. Pathological records showed pancytopenia, endocarditis, myocarditis, cardiomyopathy. We in-

TABLE 1
Results of nested PCR for detection of DNA of Ehrlichia phagocytophila *and* Borrelia burgdorferi sensu lato *in animals*

source	Total No.	Positive *E. phagocytophila*	Positive HGE	No. positive in %	PCR positive *B. garini*	PCR positive *B. burgdorferi*	No. positive in %
Horses, import	38	1	3	4 (10.5)	1	0	1 (3.8)
Capreolus capreoli	39	5	1	6 (15.3)	3	1	4 (19.0)
Sus scrofa	38	4	1	5 (13.6)	1	1	2 (5.2)
Vulpes vulpes	6	1	0	1 (16.6)	0	0	0
Cervus elafus	4	1	0	1	0	0	0
Dama dama	8	1	0	1 (12.5)	0	2	2 (25.0)
Larus ridibundus	9	1	0	1 (11.1)	2	1	3 (33.3)
Anas platyrhinchos	10	1	0	1 (10.0)	2	0	2 (20.0)
Phasianus colchicus	12	2	0	2 (16.6)	4	1	5 (41.6)
Phalacrocorax carbo	1	1	0	1	0	0	0
Garrulus glandarius	6	0	0	0	1	0	1 (16.6)
Corvus frugilegus	10	0	0	0	3	0	3 (30.0)
Apodemus sylvaticus	25	2	0	2 (8.0)	2	0	2 (13.3)
Microtus arvalis	17	2	0	2 (11.7)	1	4	5 (29.4)
Clathrionomys glareo-	13	1	0	1 (7.6)	2	0	2 (25.0)
Lus Ixodes ricinus	90	4	2	6 (10.6)	6	2	8 (13.3)
Lipoptera cervi	22	1	0	1 (4.5)	1	0	1 (4.5)

vestigated autopsy specimens of the brain, kidney and heart for elimination of *Borrelia* infection. Due to the reported pancytopenia, we processed all samples also for the detection of HGE and HME agents. PCR- sequenation was positive in the kidney and the HGE agent was also detected on electron microscopic examination as shown in Figure 1 and 2. In the brain, instead of HGE we detected *Borrelia garini.* Sequencing showed 100% similarity to B.garinii, serotype 4 (GenBank, No. X80256).
Patient No. 2. Coincidence of *B. afzelii* and *E. phagocytophila* (HGE) was found in the blood of a 58-year-old woman (our technician) with EM at the site from which we removed a tick nymph one week earlier (May 8, 2000). EM was accompanied by febrilia, myalgia and gastroenteritis. She was immediately treated with doxycycline. Her convalescent sera were positive in WB IgM and IgG for *B. garinii* and in IFA IgM for HGE. Nested amplification detected a 390 bp product in the course of 2 days treatment, and sequencing of the amplicon disclosed HGE agent. Cultivation of both agents was unssuccesful due to the immediate treatment.



GRAPH 1 - *Ehrlichia phagocytophyla* complex diagnosed by PCR in 10 humans and in 28 animals and *Borrelia burgdorferi sensu lato* diagnosed in 33 animals from June 2000 through December 2001 in central, west and eastern regions of the Czech republic.

Patient No. 3, the head of a neighbouring laboratory had EM on the thorax two weeks after a tick bite on April 16, 2000. He was hospitalized with myalgia and fever and was treated with doxycycline. PCR was positive from the blood before the treatment and on days 2, 5, 7 of his illness. Acute sera had IgM IFA titer ≥32. Convalescent sera were PCR negative but the IgG IFA titre was 1:520. Hematological examination revealed marked leukocytosis and a low number of thrombocytes. Culture with HL-60 cells was positive before treatment and on day 2 but coccobacilli stayed alive only to the 3rd passage. Aliquots from the passage were kept at –80°C. Coccobacilli formed clusters after 1 week of culture as revealed by negative staining (Figure 3) and in sections stained with Giemsa - Wright (Figure 4).

Clinical anamnesis from another 7 patients showed fever, myalgias and/or mild neurological symptoms after tick bite. These patients had no EM. Three PCR positive patients had IgG anti-ehrlichia HGE antibodies, two had IgM antibodies in IFA tests. Two huntsmen were PCR positive for HGE in the blood, had myalgia and negative serology against HGE.

The partial 16S rRNA gene sequence of patient No. 2 was 100% identical with that of *Ehrlichia* sp. "Baden" (accession No. AF136714). Other patients No. 1, 3, 5, 6, had diagnosed sequences 100% identical to the HGE agent (GenBank accession No. AF189-153). One huntsman was PCR negative but IFA IgG positive for the HGE agent. Patient No. 4, transiting CZ from the USA and Thailand to London. He had many tick bites. He had fever, rash, anorexia and mental changes. He did not want to be hospitalized and asked for investigation in our laboratory. His hematological examination revealed lymphocytosis and leucocytosis, but granulocytic inclusions were not seen. A single 600 bp product was detected with the outer primers (Harrus *et al.*, 1998) and in nested PCR for 16S rRNA of *Ehrlichia* sp. Direct sequencing of the amplicons from both PCR tests revealed the presence of *E. chaffeensis*, the HME agent. He was treated in the General Hospital, Glasgow, UK. Serological results for antibodies against the HGE agent were negative and against *Borrelia burgdorferi sensu lato* were positive.

A total of 28 out of 236 animals (11.8%) from three regions of CZ were positive in nested PCR for *Ehrlichia* spp. (Table 1). A single 390 bp PCR products with primers HE3 and HE3-R (Figure 5) or 619 bp products with the primers 593II and ERB2 were visible. Peripheral blood smears obtained directly from 2

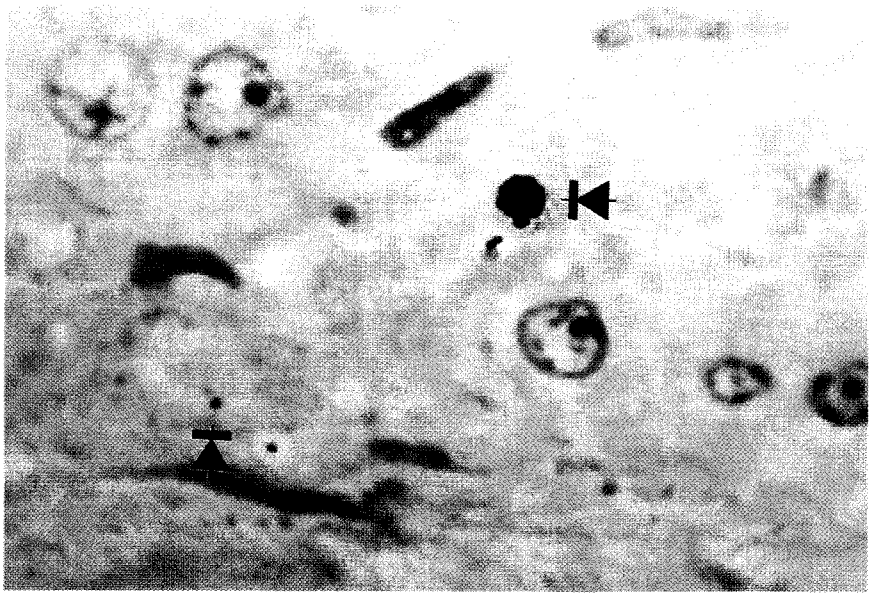


FIGURE 1 - Immunohistologic demonstration of the HGE agent morulae (arrow) in a macrophage from the kidney autopsy sample of a patient with fatal infection in Lowicryl K4M semithik section, HGE-immuno-peroxidase-DAB and Toluidine blue counterstain. Original magnification, 1000 x.

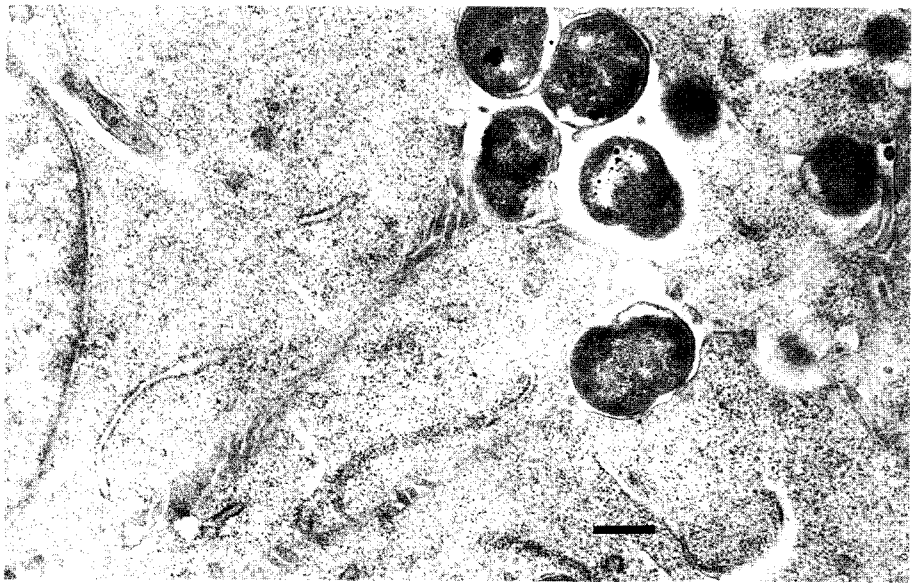


FIGURE 2 - Transmission electron micrograph of electron-dense coccobacilli in a membrane lined endosome of the macrophage. Coccobacilli in diameter 1-2 μm are delineated by a double membrane with an electron lucent space between membrane and dense core. Tranverse section of the kidney sample in Uranyl acetate, Lead citrate staining. Magnification, 12 400 x. Bar, 1μm.

shootings of *Capreolus capreoli* and from *Sus scrofa* revealed granulocytic inclusions in leucocytes stained with Giemsa. The reverse hybridization assay with *E. equi* probe and HGE probe in ELISA plates showed 4 *Capreolus capreolus* and *2 Sus scrofa* specimens positive with similar absorbance as was in the control (Figure 6). Three out of 6 PCR amplicons from roe deer showed sequences homologous with the Frankonia I variant with a G instead of an A nucleotide at position 76 and a A instead of an G at position 84, two with *Ehrlichia* sp. "Baden" and one with *E. equi*. Four PCR positive horses had *E.equi* with sequence identity to the HGE agent. Sequencing of 390 bp products of isolated DNA from tissue sam-

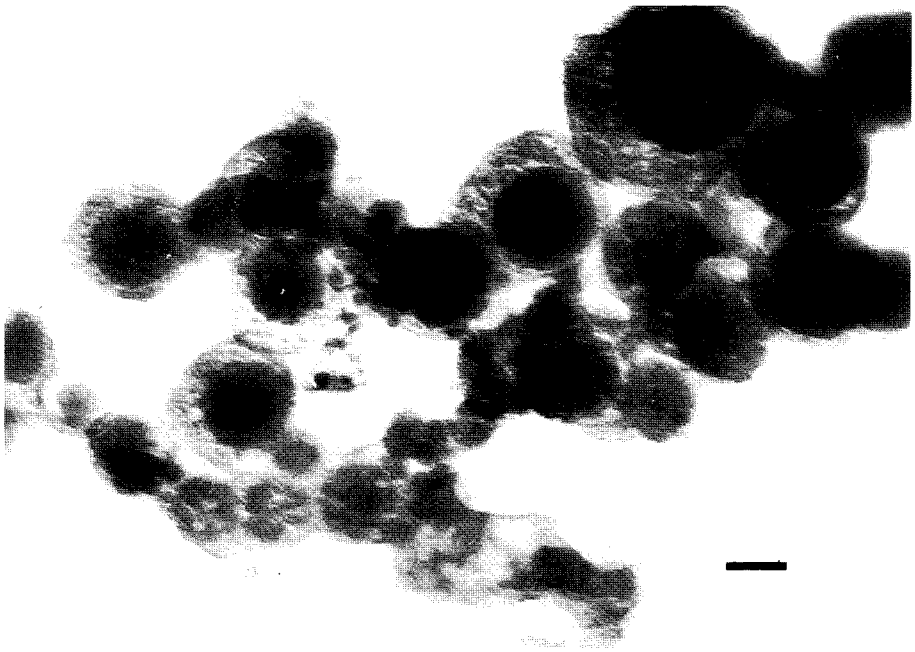


FIGURE 3 - Transmission electron micrograph of native HGE agents spilling from ruptured cells into PBS supernatant. Negative staining with 1% PTA. Magnification, 49 000 x. Bar, 0,5μ.

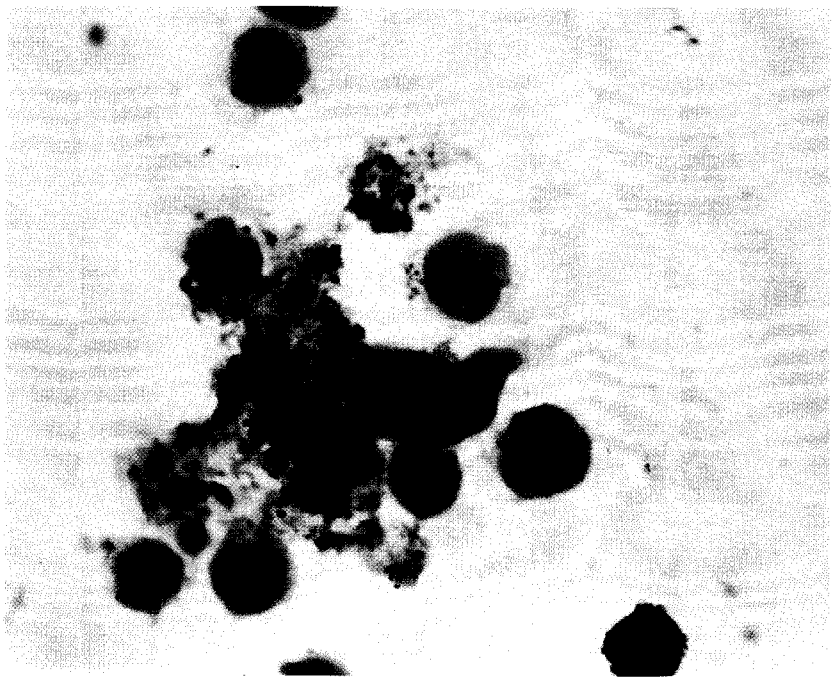


FIGURE 4 - Light micrograph of HGE agents in the cytoplasm or attached on the surface of HL-60 cells in 2nd passage. Giemsa-Wright staining. Magnification, 1290 x.

ples (kidney or spleen) of 5 out of 38 boars (*Sus scrofa)* disclosed two variants of *E. phagocytophila*: variant "Baden" with 99.9 % identity (with a G instead of an A at position 76), variant Frankonia II with 99% identity (with a T instead of an C at position 124). PCR positive samples from one moufflon had sequence identity with a variant isolated from *Lama glama* (accession No. AF241532). None of the *E. phagocytophila* cultures were obtained by inoculation of tissue samples that were PCR positive, into HL-60 cells. Cultures were lost due to contamination. Sequencing of some tissue samples from *Sus scrofa* and roe deer revealed *Pseudomonas* sp. SW2 and *Brachyspira hyodisenteriae* (accession No. AF 395882).

The muscle and heart samples from shooting birds were predominantly infected with *B. garini* (Table 1) and only in 6 of them was there obtained a single 390 bp product in nested PCR for the 16S rRNA gene of *E. phagocytophila.* PCR products from muscle specimens of *Phalacrocorax carbo, Phasianus colchicus* and from *Columba palumbus* were analysed a variant of *Ehrlichia phagocytophila* with 99% identity to variant Frankonia I. The other PCR- positive birds, as were *Anas platyrhynchos* and *Larus ribidindus* had more than 2 bands in nested PCR due to contamination with other bacteria rendering sequencing impossible. Hybridization with *E. equi* probes in ELISA was also negative.

Murine kidney and spleen specimens collected from 55 mice caught in Moravia and in a horse breeding farm were infected with *Borrelia burgdorferi sensu lato* (16.3%) and with *E. phagocytophila* or the HGE agent in 9%. Ticks collected from eastern regions were infected in 6.6 % with HGE or *E.equi* and in 8.8 % with *B. burgdorferi sensu lato* as shown in Table 1.

DISCUSSION

In the present study, we used PCR with sequence analysis or hybridization of products to detect and identify *Ehrlichia sp.* and *B. burgdorferi sensu lato* in some Czech patients and animals. Analysis revealed infection rates in animals with *Ehrlichia sp.* in 11.8 % and with *B. burgdorferi sensu lato* in 13.9%. Both agents are being increasingly detected in three

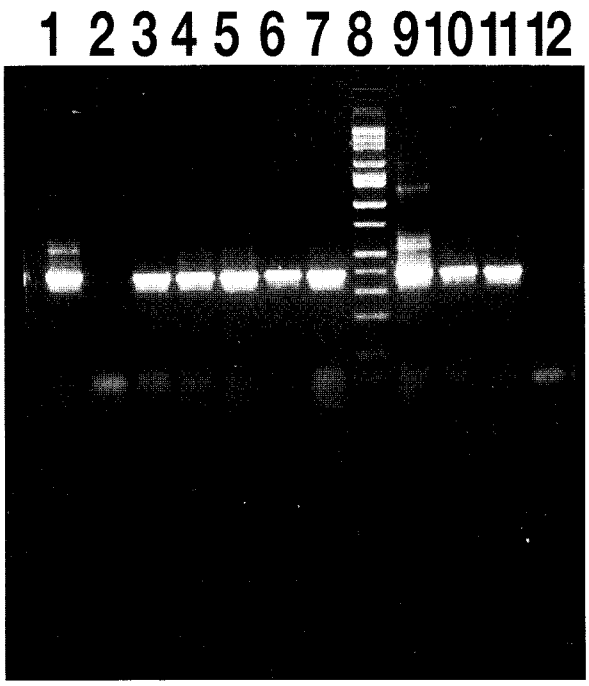


FIGURE 5 – Agarose gel of *E. phagocytophila* DNA fragments amplified with inner primers HE3, which give 390 bp specific fragment for DNA of granulocytic ehrlichiae. Target DNAs were amplified from the folowing: lane 1 - positive control HGE in HL-60 cells, lane 2 – negative control, HL-60 cells, lanes 3-7 show the aspected 390 bp products from DNA samples from roe deer, lane 8 – PCR low molecular marker (Sigma), lane 9 – positive control, native HGE in PBS, lane 10-11 – products from target DNAs from boar samples, lane 12 – PCR negative control, nested amplification mixture without DNA.

geographic regions, west, central and east of CZ where *Ixodes ricinus* ticks are endemic. In Europe, the *Ixodes ricinus* tick is known as a vector of tick-borne fever of animals (Rikihisa, 1997). An *Ehrlichia* strain identical to the HGE agent has been identified in Swedish dogs and horses (Johansson *et al.*, 1995). Pancholi *et al.*, (1995) found that 12% of 25 sera from patients with borreliosis had antibodies against *E. equi.* Mitchel *et al.*, (1996) reported immunoserologic evidence of coinfection of HGE with *Borrelia burgdorferi* and *Babesia microti* in residents of Wisconsin. We proved coinfection in 3 patients who had EM. We found antibodies against *E. phagocytophila* and *E. equi* in 10 patients, 2 of whom had neuroborreliosis. A similar observation has been made by Lebech *et al.* (1998). Christova and Dumler (1999), detected anti-HGE antibodies in 14 of 147 patients with EM. *Ehrlichia equi* was originally reported as a cause of granulocytic infection in horses. Chen *et al.* (1995), demonstrated that sequencing analysis of the HGE agent is nearly identical to the known sequence of *E. equi.* Belongia *et al.* (1997), showed prevalence of HGE or *E. equi* in 15% PCR positive white–tailed deer. We detected infection with *E. equi* in horses, boars (*Sus scrofa)* and in roe deer. Infection with LGE has been found by Barlough *et al.* (1997). A high infection rate of the ticks (45%) collected from roe deer which may be a reservoir for

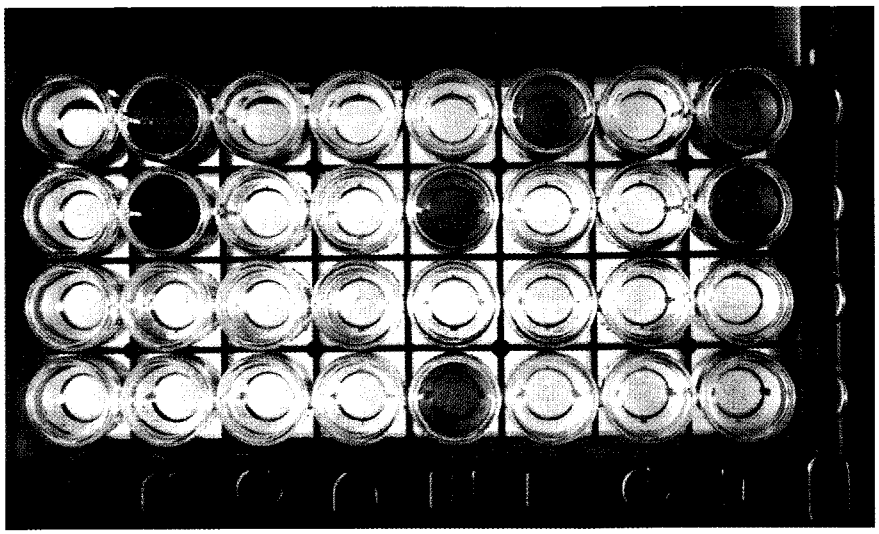


FIGURE 6 - Enzyme linked immunosorbent-hybridization assay with covalently coupled probe E.equi to the activated DNA –bind plate wells. Hybridization of the biotin-labelled PCR products from 17 samples of roes yielded absorbance in the wells designated B3,E1,E3 and F4 as in the controle in B4 well. The G and H wells represent board-derived PCR products hybridization with E.Equi probe in the G1, H3 and H4 wells. (Streptavidin-peroxidase, DAB counterstain).

Ehrlichia has been published by Schouls *et al*, (1999). Analysis of PCR products from roe deer showed: HGE and two variants of *E. phagocytophila* that carried a substitution of a single base pair of the 16S rRNA gene. The same observation was made in infected ticks from the United States (Belongia *et al.*, 1997) and from Germany (Baumgarten *et al.*, 1999). It is possible that HGE and the variant *E. phagocytophila* are the same subspecies and may be pathogenic for humans. Sequencing analysis of PCR products from our positive patients showed the HGE agent except for case No. 2 where *E. phagocytophila* subspecies "Baden" was detected. Our study included one patient who had a specimen positive for HGE by PCR-sequenation 3 months after treatment of EM, and died 5 months after the tick bite. Possible persistence of HGE infection showed Ramsey *et al.* (2002). Severe and lethal HGE infections have been described in immunocompromised patients by Safdar *et al.* (2002). Clinical and laboratory characteristics of HGE infection of humans have been shown by Bakken *et al.* (1996). One of our patients (No. 4) acquired *E. chaffeensis* infection in the USA or Thailand. This later species was not detected in our present study investigating animals and ticks. Coinfections of ticks with multiple organisms and transmission of more than 2 agents have been documented. Human exposure to granulocytic *Ehrlichiae* species and coinfection with *Borrelia spp.* occur in the Czech Republic. Reports of unusual infections of birds with *E. phagocytophila* together with *B. garinii* have not been published yet. Sequence analysis of the 16S rRNA gene demonstrated variant *Frankonia* I in *Phalacrocorax carbo, Columba palumbus* and in one *Phasianus colchicus.*

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REFERENCES

ANDERSON, B.E., DAWSON, J.E., JONES, D.E., and WILSON, K.H. (1991). Ehrlichia chaffeensis, a new species associated with human ehrlichiosis. *Journal of Clinical Microbiology*, **29**, 2838-2842.

BAKKEN, J.S., KRUETH J., WILSON-NORDSKOG, C., TILDEN, R.L., ASANOVICH, K., and DUMLER, S.J. (1996). Clinical and laboratory characteristics of human granulocytic Ehrlichiosis. *JAMA*, **275**, 199-205.

BARLOUGH, J.E., MADIGAN, J.E., TUROFF, D.R., CLOVER, J.R., SHELLY, S.M., and DUMLER, J.S. (1997). An Ehrlichia strain from a Llama (Lama glama) and Llama associated ticks (Ixodes pacificus). *Journal of Clinical Microbiology*, **35**, 1005-1007.

BAUMGARTEN, B.U., ROLLINGHOFF, M., and BOGDAN, C.H. (1999). Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic *Ehrlichiae* in *Ixodes ricinus* ticks from Southern Germany. *Journal of Clinical Microbiology*, **37**, 3448-3451.

BELONGIA, E.A., REED, K.D., MITCHELL, P.D., KOLBERT, CH.P., PERSING, D.H., GILL, J.S., and KAZMIERCZAK, J.J. (1997). Prevalence of granulocytic Ehrlichia infection among white-tailed deer in Wisconsin. *Journal of Clinical Microbiology*, **35**, 1465-1467.

BREITSCHWERDT, E.B., HEGARTY, B.C., AND and HANCOCK, S.J. (1998). Sequenation evaluation of dogs naturally infected with *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia equi, Ehrlichia ewingii*, or *Bartonella vinsonii*. *Journal of Clinical Microbiology*, **36**, 2645-2651.

BROUQUI, P., DUMLER, J.S., LIENHART, R., BROSSARD, M., and RAOULT, D. (1995). Human granulocytic ehrlichiosis in Europe. *Lancet*, **346**, 782-83.

CHEN, S.M., DUMLER, J.S., BAKKEN, J.S., and WALKER, D.H. (1994). Identification of a granulocytotropic Ehrlichia species as the etiologic agent of human Disease. *Journal of Clinical Microbiology*, **32**, 589-595.

CHRISTOVA, I.S. and DUMLER, J.S. (1999). Human granulocytic ehrlichiosis in Bulgaria. *American Journal of Tropical Medicine and Hygiene*, **60**, 58-61.

DUMLER, J.S., DAWSON, J.E., and WALKER, D.H. (1993). Human ehrlichiosis: hematopathology and immunohistologic detection of *Ehrlichia chaffeensis*. *Human Pathology*, **24**, 391-396.

DANIELS, TJ., BOCCIA, T.M., VARDE, S., MARCUS, J., BUSCHER, D.J., FALC, R.C., and SCHWARTZ, I. (1998). Geographic risk for Lyme disease and human granulocytic ehrlichiosis in southern New York state. *Journal of Clinical Microbiology*, **64,** 4663-4669.

ENGVALL, E.O., PETTERSSON, B., PERSSON, M., ARTURSSON, K., and JOHANSSON, K. (1996). A 16S rRNA PCR assay for detection and identification of granulocytic Ehrlichia species in dogs, horses, and cattle. *Journal of Clinical Microbiology*, **34**, 2179-2174.

GOODMAN, J.L., NELSON, C., VITALE, B., MADIGAN, J.E., DUMLER, J.S., KUTTI, T.J., and MUNDERLOH, U.G. (1996). Direct cultivation of the causative agent of human granulocytic ehrlichiosis. *New England Journal of Medicine*, **334**, 209-215.

HARRUS, S., WANNER, T., AIZENBERG, I., FOLEY, J.E., POLAND, A.M., and BARK, H. (1998). Amplification of ehrlichial DNA from dogs 34 months after infection with *E. canis*. *Journal of Clinical Microbiology*, **36**, 73-76.

HEIMER R., ANDEL, A.V., WORMSER, G.P., and WILSON, M.L. (1997). Propagation of granulocytic *Ehrlichia spp.* from humans and equine sources in HL-60 cells induced to differentiate into functional granulocytes. *Journal of Clinical Microbiology*, **1997**, 923-927.

HULINSKA, D., VOTYPKA, J., and VALESOVA, M. (1999). Persistence of *Borrelia garinii* and *Borrelia afzelii* in patients with *Lyme arthritis*. *Zentralblatt fur Bakteriologie*. **289**, 301-318.

HULINSKA, D., KURZOVA, Z., DREVOVA, H., and VOTYPKA, J. (2001). First lessons from Ehrlichiosis confirmed serologically and by PCR analysis in patients with Borreliosis in the Czech Republic. *(In Czech) Journal of the Czech Physicians*, **6**, 181-184.

JOHANSSON, K.E., PETTERSON, B., UHLEN, M., GUNNARSON, A., MALMQVIST, M., and OLSSON, E. (1995). Identification of the causative agent of granulocytic ehrlichiosis in Swedish dogs and horses by direct solid phase sequencing of PCR products from 16S rRNA gene. *Res. Veterinary Science*, **58**, 109-112.

LEBECH, A.M., HANSEN, K., PANCHOLI, P., SLOAN, L.M., MAGERA, J.M., and PERSING, D.H. (1998). Immunoserologic evidence of human granulocytic ehrlichiosis in Danish patients with *Lyme neuroborreliosis*. *Scandinavian Journal of Infectious Diseases*, **30**. 173-176.

LIZ, J.S., ANDERES, L., SUMNER, J.W., MASSUNG, R.F., GERN, L., RUTTI, B., and BROSSARD, M. (2000). PCR detection of granulocytic Ehrlichiae in *Ixodes ricinus* ticks and willd small mammals in western Switzerland. *Journal of Clinical Microbiology*, **38**, 1002-1007.

MARCONI, R.T., HOHENBERGER, S., SPECHTEL, S., LAVORE, C.P., ROBLER, D., and WILSKE, B. (1999). Genetic analysis of *Borrelia garinii* OspA serotype 4 strains associated with neuroborreliosis, evidence of extensive genetic homogeneity. *Journal of Clinical Microbiology*, **37**, 3965-3968.

MITCHELL, P.D., REED, K.D., and HOFKES, J.M. (1996). Immunoserologic evidence of coinfection with *Borrelia burgdorferi*, *Babesia microti*, and human granulocytic Ehrlichia species in residents of Wisconsin and Minnesota. *Journal of Clinical Microbiology*, **34**, 724-727.

MASSUNG, R.F., MAUEL, M.J., OWENS, J.H., COURTNEY, J.W., STAFFORD, K.C., MARTHER, T.N. (2002). Genetic variants of *Ehrlichia phagocytophila*, Rhode Island and Connecticut. *Emerging Infectious Diseases*, **8**, 467-472.

PANCHOLI, P.,KOLBERT, C.P., MITCHELL, P.D., REED, K.D., DUMLER, J.S., BAKKEN, S.R., TELFORD, S.R., and PERSING, D.H. (1995). *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. *Journal Infection Diseases*, **172**, 1007-1012.

SCHOULS, L.M., VAN DE POL, I., RIJPKEMA, G.T., and SCHOT, C.S. (1999). Detection and identification of *Ehrlichia*, *Borrelia burgdorferi sensu lato* and Bartonella species in Dutch *Ixodes ricinus* ticks. *Journal of Clinical Microbiology*, **37,** 2215-2222.

RAMSEY, A.H., BELONGIA, E.A., GALE, C.M., and DAVIS, J.P. (2002). Outcomes of treated human granulocytic ehrlichiosis cases. *Emerging Infectious Diseases*, **8**, 398-401.

RIKIHISA, Y., ZHI, N., WORMSER, G.P., WEN, B., HOROWITZ, H.W., and HECHEMY, K.E. (1997). Ultrastructural and antigenic characterization of granulocytic ehrlichiosis agent directly isolated and stably cultivated from patient in New York State. *The Journal of Infectious Diseases*, **175**, 210-213.

SAFDAR, N., LOVE, R.B., and DENNIS, G.M. (2002). Severe *Ehrlichia chaffeensis* infection in a lung transplant recipient: a review of ehrlichiosis in the immunocompromised patient. *Emerging Infectious Diseases*, **8**, 320-323.

VASILIU, V., HERZER, P., ROSSLER, D., LEHNERT, G., and WILSKE, B. (1998). Heterogeneity of *Borrelia burgdorferi sensu lato* demonstrated by an OspA- type –specific PCR in synovial fluid from patients with Lyme arthritis. *Medical Microbiology, Immunology*, **187,** 97-102.

WALKER, D.H., and DUMLER, J.S. (1997). Human monocytic and granulocytic ehrlichiosis. *Archiv Pathological Medicine*, **121**, 785-791.

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