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Published in:
Clinical and Diagnostic Laboratory Immunology

DOI:

2002

Citation for published version (APA):

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Isolation and Characterization of Two European Strains of *Ehrlichia phagocytophila* of Equine Origin

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Received 20 March 2001/Returned for modification 7 June 2001/Accepted 24 October 2001

We report the isolation and partial genetic characterization of two equine strains of granulocytic *Ehrlichia* of the genogroup *Ehrlichia phagocytophila*. Frozen whole-blood samples from two Swedish horses with laboratory-verified granulocytic ehrlichiosis were inoculated into HL-60 cell cultures. Granulocytic *Ehrlichia* was isolated and propagated from both horses. DNA extracts from the respective strains were amplified by PCR using primers directed towards the 16S rRNA gene, the groESL heat shock operon gene, and the ank gene. The amplified gene fragments were sequenced and compared to known sequences in the GenBank database. With respect to the 16S rRNA gene, the groESL gene, and the ank gene, the DNA sequences of the two equine *Ehrlichia* isolates were identical to sequences found in isolates from clinical cases of granulocytic ehrlichiosis in humans and domestic animals in Sweden. However, compared to amplified DNA from an American *Ehrlichia* strain of the *E. phagocytophila* genogroup, differences were found in the groESL gene and ank gene sequences.

Granulocytic *Ehrlichia* causes febrile diseases in many different animals and in humans. The first human cases were described for the United States in 1994, but clinical cases are now accumulating from many countries, mainly in temperate regions (3, 24, 29; A. van Dobbenburgh, A. P. van Dam, and E. Fikrig, Letter, N. Engl. J. Med. 340:1214–1216, 1999). In Scandinavia, clinical granulocytic ehrlichiosis (GE) has been reported for humans, cattle, sheep, horses, dogs, and cats (5, 7, 14, 26). The infectious agents are strictly intracellular life in the wildlife reservoir and the infected host, as well as prolonged survival in the principal vector, hard-bodied ticks of the genus *Ixodes*. Until the successful isolation of granulocytic ehrlichias in HL-60 cells, the possibilities of studying these bacteria were limited to indirect and molecular biological methods (16). Today, reports of successful isolation of granulocytic *Ehrlichia* from humans and animals are available from the United States but not from other parts of the world. The aim of this study was to isolate, to maintain in culture, and genetically characterize European strains of granulocytotropic *Ehrlichia phagocytophila*. Moreover, earlier reports on the isolation of granulocytic *Ehrlichia* spp. were based only on isolation from fresh blood. In this paper, we report the isolation of European *E. phagocytophila* of equine origin from stored frozen whole blood.

**MATERIALS AND METHODS**

Patients. Two horses (a 4-month-old Swedish trotting horse and a 21-year-old pony from southwest Sweden) entered the Halland Animal Hospital, Söinge, Sweden, in the fall of 1998 with fever, malaise, and anorexia. EDTA-blood was collected under sterile conditions and frozen at 20°C without further prepara-

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Blood smears showed cytoplasmic inclusions in approximately 25 to 30% of the neutrophils in both cases, supporting the clinical diagnosis of GE. Both horses were treated with intravenous oxytetracycline (7 mg/kg of body weight daily for 7 days) and recovered clinically within 24 h.

**Culture of ehrlichias in HL-60 cells.** Promyelocytic HL-60 leukemia cells (ATCC CCL240) were maintained in antibiotic-free RPMI 1640 medium, supplemented with 2 mM l-glutamine and 20% fetal bovine serum. The HL-60 cells were incubated at 37°C in an atmosphere of 5% CO2 (16). An aliquot of 0.5 ml of the sediments leukocyte-rich fraction of equine EDTA-blood (kept at 20°C for 7 months) was inoculated into 25-cm² flasks with HL-60 cells at a density of 2 × 10⁵ cells/ml. The infected cells were then monitored daily by microscopy and examination of Giemsa-stained cryopok-prepared cell spreads. The cultures were kept at a density of 2 × 10⁵ to 4 × 10⁵ cells/ml by feeding them with medium two to three times a week. Infection of the cells was quantified by the presence of morulae and by indirect immunofluorescence assay using a bovine anti- *Ehrlichia* immunoglobulin G-positive serum and a fluorescein isothiocyanate-conjugated goat anti-bovine immunoglobulin G antibody (product no. 209-095-088; Jackson ImmunoResearch Laboratories), diluted 1:100 in phosphate-buffered saline, as secondary antibody. Noninfected HL-60 cells were used as negative controls.

**PCR amplification and sequence analysis.** DNA was extracted from infected and noninfected cells with the Qiagen Tissue Kit protocol (Qiagen GmbH, Hilden, Germany). Nested PCR protocols targeting the 16S rRNA gene, the ank gene, and the groESL gene were followed as described previously, but with minor changes (21, 22, 27). The primers used to amplify the 16S rRNA gene were 16SF1 (5′ AGAGTTTGTATCCGCTGCT) and GE10 (5′ TCCGTTAAGAGATCTAATCTCC) for the primary reaction and EC12A (5′ TGATCCTGGCTCAGAAGCAAGC) and EHR790 (5′ CTTAACCCGTAGCTACAAACAG) for the nested reaction. In the groESL assay, HS43 (5′ ATAGCTAAGGAGCATAGTGC) and HS45 (5′ ACTTCAGGTCTCTAGACG) were used for the primary reaction, and GEH91 (5′ AGCTCTAGCTAGCTTAGT) and EBS6 (5′ ACAAATACAGCTCCAGCA) for the nested reaction. In the ank gene assay, AQF23 (5′ GAAGAAATTTACAACCTGTAAGAG) and AQ2R2 (5′ CGACCAATCGATGAACGTTTCACTG) were used for the primary reaction, and AQF25 (5′ TTGACCCGTAGAAGCATTACAC) and AQ2R1 (5′ ACCATTGCTTCTGAGAG) for the nested reaction. Each of the primers was subsequently used for direct sequencing of the appropriate purified PCR product. Two additional primers were needed to sequence the 781 bp region amplified by the 16S rRNA PCR, EB4 (5′ GTATTACCACCGCGCTGCTGCAGC) and EHR521 (5′ GTAGAAGCGTGTCCGTAAGTGAAGAG) for the nested reaction.

Sequencing reaction products were separated and analyzed using an automated sequencer (ABI 377; Applied Biosystems) and fluorescent-labeled dideoxynucleotide technology. Sequences were edited and analyzed with the Staden software.
The two strains showed similar propagation patterns. Seven days after equine blood and HL-60 cells were mixed, the first signs of morulae could be noted in the infected cells when they were analyzed with Giemsa-stained cytospin preparations (Fig. 1). Noninfected cells, grown and analyzed in parallel, showed no corresponding cell changes. During the following days, the extent of infection increased: 10% infected cells at day 7 and 60% at day 16. After day 16, the infection declined so that at day 26 only 20% of the cells were infected.

Cells infected with the respective strain were positive in PCR assays targeting the 16S rRNA gene, the ank gene, and the groESL gene of granulocytic Ehrlichia. The amplified 781-bp fragments of the 16S rRNA gene sequences were identical to the sequences obtained from our equine E. phagocytophila isolates were identical to each other and to a previously characterized Swedish E. phagocytophila isolate from an infected human but differed from all other Ehrlichia groESL sequences present in GenBank by at least two nucleotides (27).

The principal wildlife reservoir of E. phagocytophila is believed to be small mammals, mainly rodents, and deer (1). The fact that both the reservoir and vector species differ between North America and Europe and that coevolution of the bacteria, the vector, and the host must to a large extent be independent processes on the two continents means that significant differences in strain characteristics can be expected, both genetically and in terms of phenotypic traits, such as antigenic profile, host preferences, and virulence. Antigenic pleomorphism has been reported earlier for various isolates of E. phagocytophila (2, 20, 30). Our results show differences be-
between the North American and European variants of *E. phagocytophila* and suggest that the ank gene provides useful information complementary to that from the 16S rRNA gene that can be used to divide *E. phagocytophila* into clades corresponding to geographic distribution (9). This is interesting, as some of the differences found may lead to better geographically adapted diagnostic tools and to understanding of differences in bacterial virulence and host preferences (2, 20, 23, 28, 30). Thus, these results warrant further comparisons of *E. phagocytophila* strains of different geographic origins in terms of genetic relationships, expression of antigens, ecology, and epidemiology.

**ACKNOWLEDGMENTS**

This study was supported by grants from the Intervet Veterinary Research Foundation (981116), the Health Research Council of Southeast Sweden (F98-118), and the Swedish National Board for Laboratory Animals (CFN, Dnr 00-41).

We thank Gunvor Johansson at the Halland Animal Hospital for expert technical assistance in collecting samples and J. Stephen Dumler for helpful advice.

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