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

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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 rickettsia-like bacilli, with the capacity for 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 granulocytotrophic *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, Slöinge, 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 preparation. Additional blood was collected and investigated by direct microscopy. 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, in an aliquot of 0.5 ml of the sedimented leukocyte-rich fraction of equine EDTA-blood (kept at 20°C without further preparation, and refrigerated within 24 h).

**PCR amplification and sequence analysis.** DNA was extracted from infected and noninfected cells with the QIAamp 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 16SFL (5′ AGAGTTTGTATCTGCTGGTAC) and GE10 (5′ TCCGTTAAAGGATCTCTACTCC). The primary reaction 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 cytoplasmic-prepared cell spreads. The cultures were kept at a density of 2 × 106 to 4 × 107 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. 209-088; Jackson ImmunoResearch Laboratories), diluted 1:100 in phosphate-buffered saline, as secondary antibody. Noninfected HL-60 cells were used as negative controls.

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signs of ehrlichiosis and responded well to treatment. Thus, the
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FIG. 1. Photomicrograph of *E. phagocytophila*-infected HL-60 cells stained with Giemsa stain. Magnification, approximately ×900.

RESULTS

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 cytoospin preparations (Fig.
1). Noninfected cells, grown and analyzed in parallel, showed
no corresponding cell changes. During the following days, the
time 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 iden-
tical to corresponding sequences of amplified DNA obtained
in Swedish human cases of GE (19). Similarly, the *groESL* gene
and the *ank* gene sequences of the equine *Ehrlichia* isolates
were identical for the two isolates and identical to the se-
quences of the corresponding gene fragments from Swedish
human strains (21).

DISCUSSION

GE in horses was described as a clinical entity in Scandinavia
in 1990 (6). The two infected horses in the present study resided in an area in southern Sweden where GE is commonly
diagnosed in horses and dogs. They showed typical clinical
signs of ehrlichiosis and responded well to treatment. Thus, the
two *Ehrlichia* isolates described represent pathogenic granulo-
cytic *Ehrlichia* strains from an area of southern Sweden where
GE is endemic. To our knowledge, these two strains of equine
origin are the first two European granulocytic *Ehrlichia* strains
to be isolated and propagated in cell culture.

As gene sequence data for granulocytic ehrlichias accumu-
late, proposals have been made to merge *E. equi*, *E. phago-
cytophila*, and the human GE agent into a single species, *E.
phagocytophila* (10, 13, 27). In accordance with these proposals
and with data obtained in this study, *E. phagocytophila* has
been used to designate the clinical isolates in this study.

The establishment of an infection in a cell line may vary in
time and rate. In this study 10% of the HL-60 cells were
infected 1 week after infection with the two equine isolates.
Other studies of in vitro cultures of *E. phagocytophila* in HL-60
cells have shown that some culture systems result in an infec-
tion rate of ≥50 to 60% while other systems never reach more
than a 2 to 5% infection rate. The infections are also estab-
lished with different rapidities (16). These differences probably
relate to the bacterial load of the inoculum, to *Ehrlichia* strain
variations, and to variations in the HL-60 cell populations.

The 16s rRNA gene has become the “gold standard” for
classification of bacteria. In this study, the nucleotide se-
quences of the 781-bp fragments of the 16S rRNA gene of the
equine *E. phagocytophila* isolates were completely identical to
the most common sequence variant of 16S ribosomal DNA
obtained in clinical cases of GE in humans, cattle, horses, dogs,
and cats in Scandinavia and other parts of Europe, as well as in
human and canine cases in the United States (4, 7, 15, 17–19,
24).

In order to investigate the genetic and antigenic relationship
between closely related bacterial species, the comparison of
more-variable genes, e.g., genes of structural proteins, may be
of value, since the 16S rRNA gene is too conserved to be able
to resolve strain differences at this level. One possible gene to
study is the *ank* gene, coding for a 160-kDa cytoplasmic protein
antigen (8, 25). Analyses of this gene from several granulocytic
*Ehrlichia* strains from geographically different areas resulted in
the division of *E. phagocytophila* into three distinct clades:
northeastern United States, upper midwestern United States,
and Europe (21). The *ank* gene sequences of our two equine
isolates were identical to previously described *ank* gene se-
quences in Swedish and Slovenian *E. phagocytophila* isolates
from humans and animals but showed only 94 to 96% identity
with *ank* gene sequences of North American *E. phagocytophila*
isolates. The *groESL* 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 nucle-
otide differences (27).

The principal wildlife reservoir of *E. phagocytophila* is be-
lieved 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 bac-
teria, the vector, and the host must to a large extent be inde-
pendent processes on the two continents means that significant
differences in strain characteristics can be expected, both ge-
etically and in terms of phenotypic traits, such as antigenic
profile, host preferences, and virulence. Antigenic pleomor-
phism 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 of 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.

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