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Analysis of Genetic Identity of North American *Anaplasma phagocytophilum* Strains by Pulsed-Field Gel Electrophoresis

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Biological and geographic heterogeneity of anthropozoonosis caused by *Anaplasma phagocytophilum* is poorly understood. Seven North American *A. phagocytophilum* strains were compared by PFGE. The average genome size was 1.58 Mbp, and restriction patterns were identical. New World strains of *A. phagocytophilum* have a large genome and a high degree of genetic uniformity.

The recently renamed *Anaplasma* (*Ehrlichia*) *phagocytophilum* is an obligate intracellular bacterium that causes disease in humans, horses, dogs, and ruminants (5, 7). These organisms were originally classified as distinct species because of apparent diverse hosts and relatively remote geographic distributions. However, genetic and antigenic analyses showed only minor differences among *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the human granulocytic ehrlichiosis (HGE) agent and revealed a close relationship to *Anaplasma marginale*, a ruminant erythrocyte pathogen (7). The new taxonomic assignment creates a single species but allows for biological and clinical heterogeneity, as is evident with the disparity between seroprevalence and incidence in humans and horses in North America and ruminants in Europe, in clinical severity between geographic locations in North America and Europe, and between clinical manifestations of infection in various mammalian hosts (1, 2, 4, 8–10, 11, 14, 19). The clinical and host tropism diversity suggest undiscovered differences among these bacteria. Thus, to determine whether *A. phagocytophilum* genomic variation might explain some heterogeneity, we analyzed genomic DNA of North American human, equid, and canid isolates by pulsed-field gel electrophoresis (PFGE).

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A. phagocytophilum (HGE agent and *E. equi*) strains (Table 1) were cultivated from peripheral blood in HL-60 cells and were passaged from 2 to 20 times prior to DNA preparation (3). The isolates were verified by PCR amplification and/or 16S rRNA gene sequencing and by reaction with *A. phagocytophilum* Msp2 (p44) monoclonal antibody. To preclude host cell DNA contamination, bacteria were purified from heavily infected HL-60 cells as previously described (3). Briefly, infected cells were harvested by centrifugation (500 × *g* at 4°C), resuspended in sucrose phosphate glutamine buffer (SPGn), and lysed by sonication on ice. Host cell debris was removed by centrifugation (500 × *g*), and the bacteria-enriched supernatant was treated with DNase I and RNase A (50 μg/ml) for 45 min at 37°C. Bacteria were centrifuged on discontinuous 45 and 30% meglumine diatrizoate density gradients for 1 h at 26,000 × *g*. The gradient interface was harvested and washed with SPGn. Protein concentration was determined, and suspensions were frozen at −80°C until they were used.

Thawed bacteria were embedded in 0.8% InCert agarose (FMC) in Bio-Rad PFGE plug molds by using sufficient bacteria to obtain 2.5 mg of protein/ml in a solution containing 10 mM Tris, 150 mM NaCl, 2 mM EDTA (pH 8.0). Genomic DNA was prepared by digestion in buffer containing 100 mM EDTA, 10 mM Tris, 1% sodium dodecyl sulfate (pH 8.0) with 1 mg of proteinase K/ml for two 24-h cycles at 37°C. The proteinase K was inactivated by three 1-h washes in a solution of 10 mM Tris (pH 8.0), 2 mM EDTA with 1 mM phenylmethylsulfonyl fluoride at room temperature and then three washes in 10 mM Tris (pH 8.0), and the plugs were finally stored in 200 mM EDTA. Genomic DNA in agarose plugs was equilibrated in restriction enzyme buffer for 1 h and was digested in 300 μl of 1× restriction enzyme with bovine serum albumin carrier, if required, overnight at an appropriate temperature. Each plug was equilibrated with 0.5× Tris-borate-EDTA (TBE) for 30 min and was applied to the gel. Various restriction enzymes were assessed for resolution and for a small number of bands, including *Asc*I, *Eag*I, *Pme*I, *Swa*I, *Sgf*I, *Sma*I, *Bss*HII, *Bcl*I, *Eco*47III, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*I, *Nsi*I, *Ssp*I, *Xba*I, *Pma*CI, *Age*I, *Pst*I, *Apa*I, *Kpn*I, *Not*I, *Nhe*I, *Spe*I, *Xho*I, *Sna*BI, *Pac*I, *Rsr*II, *Dra*I, *Pvu*I, and *Sfu*I. The restriction enzyme-digested DNAs were separated by electrophoresis by using a Bio-Rad contour-clamped homogeneous electric field system and 1.6% Seakem Gold agarose (FMC) with the following empirically determined parameters: pulse times, initial 15 s and final 70 s; voltage, 6 V/cm; run time, 26 h; buffer, 0.5× TBE. Agarose gels were stained with ethidium bromide and were visualized. Molecular sizes of bands were recorded and

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TABLE 1. Isolates, strains, sources, and original locations of *A. phagocytophilum* used for PFGE

Isolate or strain	Source	Location	Passage
NY8 (HGE agent)	Human	New York State	5
MDHGE (HGE agent)	Human	New York State	3
Webster (HGE agent)	Human	Wisconsin	8
Spooner (HGE agent)	Human	Wisconsin	7
97HE97 (HGE agent)	Human	Wisconsin	3
97E13	Dog	Minnesota	2
E. equi MRK	Horse	California	20

compared by using the Bio-Rad Molecular Analyst and Fingerprinting Software, version 1.4.1.

The restriction enzymes *Asc*I, *Eag*I, *PmeI*, *Swa*I, and *Sgf*I reproducibly produced 3 to 11 bands. Each *A. phagocytophilum* isolate had identical PFGE patterns for any one enzyme (Fig. 1). Based on the sum of the molecular sizes of all bands, the average size of the *A. phagocytophilum* group genome was 1.58 Mbp, as previously documented for the Webster strain, and was much larger than the genome sizes of any other *Anaplasmataceae* or *Rickettsiale* (15).

A high degree of diversity in clinical disease exists among strains of *A. phagocytophilum* that cause infections and disease worldwide (5, 9). The initial classification into European ruminant (*E. phagocytophila*), North American equine and canine (*E. equi*), and human or canine (HGE agent) strains belies such diversity. Although unification of these species into a single *Anaplasma* species is largely accepted, differences in infectivity and clinical disease are acknowledged (1, 4, 7, 9, 10, 13, 14, 17). Although the PFGE data presented here provide further support for the new taxonomic classification, no genetic attributes explain the biological and clinical differences.

HGE is more often described as a more severe infection in North America than in Europe (5, 9). Similarly, ruminant

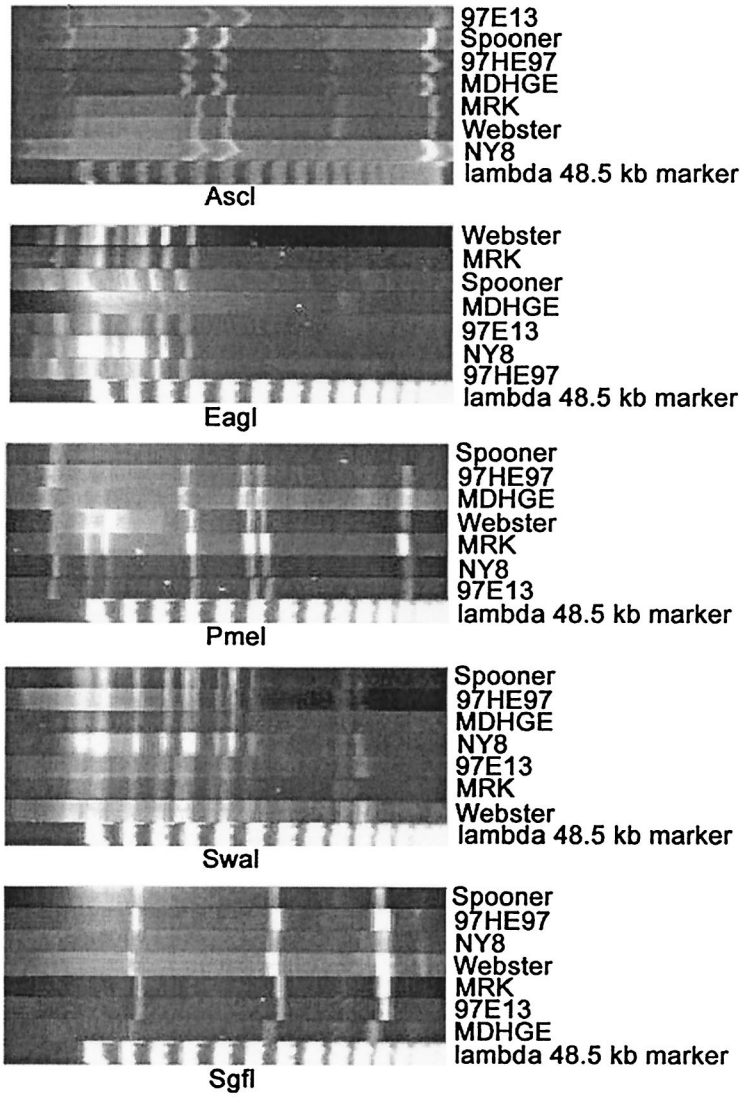

FIG. 1. Results of PFGE on seven isolates of *A. phagocytophilum* from different geographic regions in North America. The restriction enzyme used to digest the genomic DNA prepared from density gradient purified bacteria is labeled below each image. The isolate or strain of *A. phagocytophilum* is labeled on the right.

disease that is frequent in Europe is rare in North America (14, 16). Analyses of *A. phagocytophilum* genes show that North American strains differ more from European strains than they do from each other (6, 12, 18). In fact, analysis of *groESL* and *ankA* seem to confirm greater diversity among European strains, implying a longer interval of evolution that may correlate with the greater diversity of manifestations in European animals.

Complete genome sequences for many of the *Anaplasmataceae* will shortly become available, but considerable time may pass before genomic comparisons are able to provide information about diversity among organisms of a single species. The data here confirm determinations of the size of the *A. phagocytophilum* genome as well as the high degree of similarity among North American strains. Although PFGE interrogates only limited regions of the genome, it is interesting that differences in neither genome size nor restriction enzyme digestion pattern could be discerned, even among isolates from opposite sides of the continent. However, analysis by using other tools better designed to demonstrate differences, such as multilocus sequence typing, might improve detection of variations to correlate with biological behavior.

PFGE predicts that North American *A. phagocytophilum* has little heterogeneity, and potentially the reservoirs, infectious potential, and pathogenicity of this bacterium may be similar throughout North America (12, 18). A precise determination awaits further investigation of reservoirs and infections in humans and mammals with clinical signs.

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