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Molecular Characterization of *Aegyptianella pullorum* (*Rickettsiales*, *Anaplasmataceae*)

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We sequenced the 16S rRNA and *groEL* genes of *Aegyptianella pullorum*, a small bacterium that infects and replicates only in avian red blood cells. A specific PCR test was developed to analyze *A. pullorum* DNA. Phylogenic analysis revealed *A. pullorum* is most closely related to *Anaplasma* spp.

In 1928, Carpano (6) first described an infectious agent that induced intraerythrocytic inclusions in blood smears of domestic fowls in Egypt and named it *Aegyptianella pullorum*. This bacterium is transmitted by the soft tick *Argas (Persicargus*) *persicus* (7, 15, 17). The inclusions are 0.3 to 4 μm in diameter and purple when stained with the Romanowsky method. Similar inclusions have been observed in avian red blood cells in other parts of Africa, Asia, Europe, and South and North America (8, 23, 26). A large variety of bird species appear to be susceptible to infection with this agent (8, 9, 10, 16, 21, 25). Electron microscopy analysis of the South Africa Onderstepoort strain (14) and the Rhodesia strain (3) of *A. pullorum* in chicken blood revealed that the membrane-bound inclusions contain between 1 and 26 pleomorphic cocci 0.25 to 0.4 μm long. The bacterial cytoplasm includes ribosomes and fine DNA strands and is enveloped within inner and outer trilaminar membranes (3, 14). Castle and Christensen (8) described the ultrastructure of similar organisms in wild turkeys from North America in 1985. In 1992, a strain with the proposed designation "*Aegyptianella botuliformis*" was described. This strain differs from *A. pullorum* in its ultrastructure, host bird species specificity, and tick vectors (19). In addition, there are several reports of unconfirmed species of *Aegyptianella* infecting amphibians and reptiles such as frogs, tortoises, snakes, and lizards (1, 2, 4, 11, 12, 22, 27). Currently, no laboratory isolate and no molecular or antigenic data are available for *A. pullorum* or other *Aegyptianella* species. In 1974, the eighth edition of *Bergey's Manual of Determinative Bacteriology* (24) included *A. pullorum* in the family *Anaplasmataceae*, based on its phenotypic similarity to *Anaplasma marginale* (an intracellular parasite of bovine red blood cells). However, the classification of this genus was recently redesignated as uncertain, due to the lack of molecular information (13). Therefore, in the present study, we analyzed the 16S rRNA and the *groEL* gene sequences of *A. pullorum*, in order to better characterize this group of bacteria.

Ten glass slides with Romanowsky-stained blood smears from domestic broad-breasted white turkey poults inoculated with the blood from Rio Grande wild turkeys in southern Texas (*A. pullorum* Texas strain) were obtained from a study carried out in 1983 and 1984 (8). On these slides, *A. pullorum* appears as purple compact inclusions 0.3 to 4 μm in diameter (Fig. 1). In larger inclusions, clearly defined small cocci of 0.25 to 0.4 μm that resembled those of *Anaplasma* spp. could be distinguished (Fig. 1). No other cell types contained these inclusions, and no other bacteria or parasites were visually detected within the blood smear.

The slides were extensively washed with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), and cells were scraped off with a sterile scalpel blade into a microcentrifuge tube. DNA was extracted with Chelex-100 (Bio-Rad, Hercules, Calif.). Since the DNA had been severely fragmented and very small amounts of target DNA were present, we devised six nested or seminested PCRs with 12 pairs of primers, as shown in Table 1. This approach yielded overlapping ~100-bp fragments, which we assembled to map the 607 bp of the partial 16S rRNA gene sequence. The nested touchdown PCR (18) protocol included incubation at 94°C for 3 min, followed by 10 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min, with the annealing temperature decreased by 1°C in each cycle. Samples were then subjected to 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by 7 min of extension at 72°C. The 448-bp partial *groEL* sequence was obtained by assembling four ~100-bp overlapping fragments amplified by heminested PCR with eight pairs of primers, as shown in Table 1. The PCR products were cloned, and multiple clones were sequenced on both strands (Table 1). Single unique sequences were obtained for the 16S rRNA and *groEL* genes, indicating that only one bacterial species was present in the specimen. The forward and reverse primers located on different fragments were used in PCR to further verify the structure of these genes.

DNA extracted independently from different blood smear slides several months later yielded the identical sequences, indicating that these sequences were derived from the blood smear. Of note, it is unlikely that these sequences were derived from environmental contaminants, such as water or air, since the localization of *A. pullorum* is exclusively intracellular and these sequences are quite unique. It is also unlikely that these sequences are from our laboratory, since we have never used

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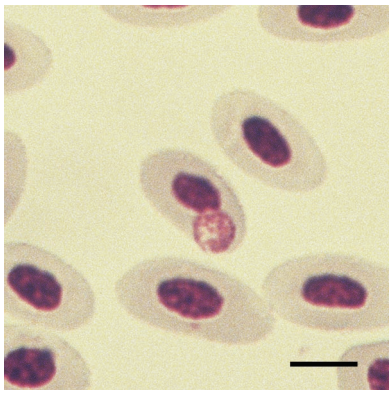

FIG. 1. *A. pullorum* inclusion in the cytoplasm of a turkey red blood cell stained with the Romanowsky method. Numerous small (<0.5-μm) reddish purple stained cocci are seen within a single round inclusion. Bar, 4 μm.

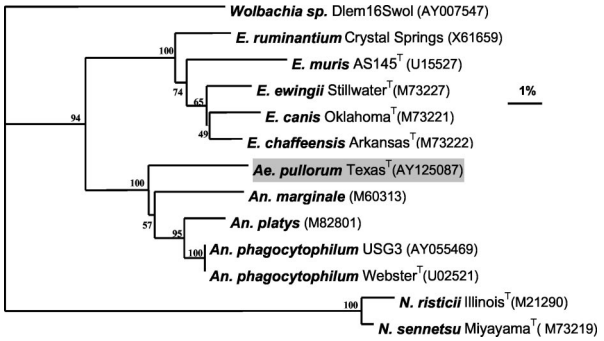

FIG. 2. Phylogram of *A. pullorum* and other members of the family *Anaplasmataceae* based on comparison of 16S rRNA gene sequences. GenBank accession numbers are shown in parentheses. Numbers above internal nodes indicate the percentages of 1,000 bootstrap replicates that supported the branch. In cases where bacterial names do not include strain names, the strain names are unavailable. Bar, percentage of divergence. The DNADIST, NEIGHBOR, CONSENSE of PHYLIP (version 3.6; http://evolution.genetics.washington.edu/phylip.html), and TreeView (version 1.5.2) programs were used for the sequence analysis and phylogram construction. Internal nodes were verified with SEQBOOT of PHYLIP with 1,000 replicates. *N. sennetsu*, *Neorickettsia sennetsu*; *N. risticii*, *Neorickettsia risticii*.

this bacterium or analyzed DNA of these base sequences. Alignment of 16S rRNA gene sequences (including the gaps) corresponding to nucleotide positions 15 to 620 of *Anaplasma marginale* and our subsequent phylogenic analysis revealed that the new sequence belonged to a member of the genus *Anaplasma* clade within the family *Anaplasmataceae* (Fig. 2). The 16S rRNA gene sequence of *A. pullorum* had 93.4, 93.2, 93.2, and 92.7% identity with the sequences of *Anaplasma platys* (strain name unavailable, an intracellular parasite of canine platelets), *Anaplasma phagocytophilum* USG3 (an intracellular parasite of human, horse, goat, mouse, and sheep granulocytes), *Anaplasma phagocytophilum* Webster^T, and *Anaplasma marginale* (strain name unavailable), respectively. These similar levels of identity indicate that *A. pullorum* is a distinct species that exists at a nearly equal distance from all known *Anaplasma* spp. Members of the next-closest clade, the genus *Ehrlichia*, had 16S rRNA gene sequences with 86.3 to 88.4% identity with that of *A. pullorum*.

Alignment of *groEL* gene sequences (including the gaps) that were available from the GenBank database corresponding to nucleotide positions 153 to 600 of *Anaplasma marginale* (strain name unavailable) and subsequent phylogenetic analysis revealed that the sequence of the *groEL* gene of *A. pullorum* was novel. This sequence was from a member of the clade that included *Anaplasma* species within the family *Anaplasmataceae* (Fig. 3). The *groEL* gene sequence of *A. pullorum* had 73.3, 72.8, and 71.4% identity with the sequences of *Anaplasma phagocytophilum* Swiss rodent, *Anaplasma phagocytophilum* WI-1, and *Anaplasma marginale* (strain name unavailable), respectively. These similar levels of identity further suggest that *A. pullorum* is at almost equal distances from *Anaplasma*

TABLE 1. Primers used for each step of nested (seminested) PCR to obtain *A. pullorum* DNA fragments and sequences and species-specific PCR[a]

Gene or procedure (bp sequenced)	First PCR			Second PCR		
	Primer pair[b]	Amplicon size (bp) without primers	Nucleotide position corresponding to *A. marginale* (M60313 or AF165812)	Primer pair	Amplicon size without primers (bp) (no. of clones sequenced)	Nucleotide position corresponding to *A. marginale* (M60313 or AF165812)
16S rRNA (607)	EU-F2 + Bird-R1	141	15–155	EU-F2 + Bird-R2	132 (9)	15–146
	EU-F1 + EU-R1	168	115–282[c]	Anap-F11 + Anap-R4	108 (7)	139–245[c]
	Bird-F1 + Anap-R2	156	203–358	Bird-F2 + Anap-R2	139 (7)	220–358
	Anap-F3 + EU-R2	105	357–461	Anap-F3 + EU-R3	87 (7)	357–443
	Bird-F3 + Anap-R7	115	414–528	Bird-F4 + Anap-R7	88 (7)	441–528
	Bird-F5 + EU-R4	103	517–620	Bird-F6 + EU-R4	91 (6)	529–620
groEL (448)	Agro-F3 + Bird-gro-R1	120	153–272	Agro-F3 + Bird-gro-R2	100 (8)	153–252
	Agro-F1 + Agro-R1	100	252–351	Agro-F1 + Agro-R2	73 (5)	252–324
	Bird-gro-F1 + Agro-R4	179	305–483	Bird-gro-F2 + Agro-R4	160 (8)	324–483
	Bird-gro-F3 + Agro-R6	138	463–600	Bird-gro-F4 + Agro-R6	118 (8)	483–600
Species-specific PCR[a]	AP-F1 + AP-R1	173[d]	50–221[c]	AP-F2 + AP-R2	113[d]	72–183[c]

[a] Species-specific primers were designed based on the newly obtained 16S rRNA gene sequence of *A. pullorum*.
[b] Primers with Bird were *A. pullorum*-specific primers; primers with Anap were designed based on conserved sequences between *Anaplasma marginale* and *Anaplasma phagocytophilum;* primers with EU were designed based on conserved sequences of 14 related *Ehrlichia* species.
[c] There is one base insertion between bases 178 and 179 corresponding to *Anaplasma marginale* (M600313).
[d] Amplicon size including primers.

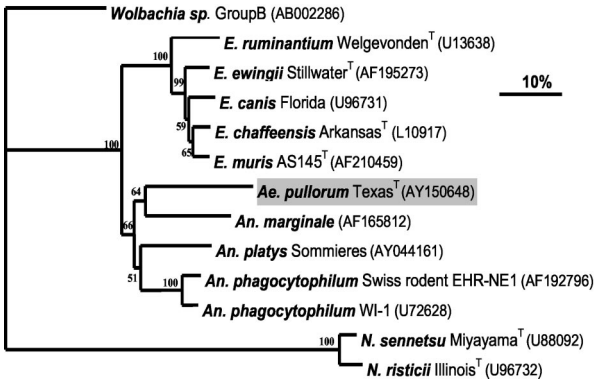

FIG. 3. Phylogram of *A. pullorum* and other members of the family *Anaplasmataceae* based on comparison of *groEL* gene sequences. GenBank accession numbers are shown in parentheses. Numbers above the internal nodes indicate the percentages of 1,000 bootstrap replicates that supported the branch. For *Anaplasma marginale*, the strain name is unavailable. Bar, percentage of divergence. Phylograms were constructed as described in the legend to Fig. 2. *N. sennetsu*, *Neorickettsia sennetsu*; *N. risticii*, *Neorickettsia risticii*.

marginale and *Anaplasma phagocytophilum*. The levels of identity of the *groEL* sequence of *A. pullorum* with the sequences of members of the next-closest clade, the genus *Ehrlichia*, were 68.3 to 69.2%.

Based on the newly obtained 16S rRNA gene sequence, we developed an *A. pullorum*-specific nested-PCR protocol. Primers for specific detection of *A. pullorum* were designed based on comparison of the *A. pullorum* 16S rRNA gene sequences with those of the most closely related species: *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia ewingii*, and *Ehrlichia canis* (Table 1). Specificities of these primers were verified by a BLAST search. In the PCR, the 50-μl reaction mixture contained a template DNA (in the second round of PCR, the template DNA used was 0.5 μl of PCR product from the first round of PCR), PCR buffer (10 mM Tris-HCl [pH 8.4], 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase, and 20 pmol of each primer. The three-step program PCR cycle conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 50 s, 54°C for 1 min, and 72°C for 1 min, and finally extension at 72°C for 7 min. Only *A. pullorum* DNA from 20-year-old slides yielded a single band of the predicted size of 113 bp (Fig. 4). In the *Anaplasma marginale* Florida and *Anaplasma phagocytophilum* HZ specimens, DNA could be amplified by a single-step PCR based on the *p44* (*msp2*) genes, as described previously (20). Neither the first-round nor the nested-PCR negative controls amplified any signals, showing

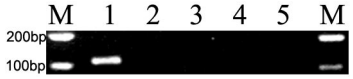

FIG. 4. Analyses of *A. pullorum*-specific PCR. Lanes 1 through 3, *A. pullorum* Texas DNA from the 20-year old slides, *Anaplasma marginale* Florida strain DNA, and *Anaplasma phagocytophilum* HZ strain DNA, respectively; lanes 4 and 5, first-step PCR and the nested-PCR template-less negative controls; lane M, 1-kb Plus DNA ladder.

that there was no DNA contamination from the environment. Eleven of the PCR product clones were sequenced, and all had sequences identical to the sequence obtained above (GenBank accession no. AY125087). These findings also indicate that this method is sufficiently sensitive to detect *A. pullorum* DNA from a 20-year-old stained blood smear on a slide.

The present molecular phylogenetic study revealed that the *A. pullorum* Texas strain is most closely related to the *Anaplasma* species, which is consistent with the previous decision to include this bacterium in the family *Anaplasmataceae* (24). This classification was based on its ultrastructure and other phenotypic characteristics. These include such observations as (i) *A. pullorum* does not multiply in cell-free media or in tissue cultures (15), (ii) attempts at continuous propagation of the organism in chicken embryos have not been successful (15), (iii) tetracyclines are effective in treating *A. pullorum* infection (24), and (iv) *A. pullorum* is transmitted by ticks (15, 17).

The 16S rRNA and *groEL* base sequences and *A. pullorum*-specific PCR developed in this study should advance our understanding of this elusive parasite in birds and ticks and facilitate the diagnosis and characterization of the diseases that are associated with it. Analysis of strains from Egypt, South Africa, and other parts of the world will clarify whether *A. pullorum* is distinct from proposed species "*A. botuliformis*" and whether it belongs to the genus *Anaplasma* or remains in a distinct genus. In general, it is extremely difficult to amplify DNA from old fixed and stained blood or tissue specimens, since such DNA is usually severely fragmented and tightly bound to dye molecules (5). Thus, the strategy and method developed in the present study may be useful for detecting other types of bacteria and their DNA sequences, in cases when fresh specimens are not readily available.

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rRNA and *groEL* gene sequences of *A. pullorum* Texas are AY125087 and AY150648, respectively.

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