

## NOTES

# Unidirectional Suppression of *Anaplasma phagocytophilum* Genotypes in Infected Lambs

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**Five-month-old lambs were simultaneously infected with different doses of two 16S rRNA genetic variants of *Anaplasma phagocytophilum* and thereafter followed for clinical observation and blood sampling. The result of the study indicates a unidirectional suppression of genotypes in infected lambs, at least during a certain period of an *A. phagocytophilum* infection.**

The rickettsia *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*) causes tick-borne fever in domestic ruminants, a disease which has also been diagnosed in several other animal species and human beings (2, 10, 23). In Europe, *A. phagocytophilum* is mainly transmitted by *Ixodes ricinus* ticks.

Based on sequencing of the 5' part of the *A. phagocytophilum* 16S rRNA gene, four genetic variants have been identified in sheep (16). Although the variants can be distinguished in the laboratory on the basis of only 1 or 2 nucleotide differences in the 16S rRNA gene, studies indicate that there are biological, ecological, and pathological differences between them (11, 18).

Simultaneous infections of several *A. phagocytophilum* 16S rRNA gene variants have been detected in individual lambs (16). A recent study indicated a possible interaction between these genetic variants (19). In the present study, susceptible lambs were simultaneously infected with various doses of two 16S rRNA variants in order to monitor further the occurrence of variants during an *A. phagocytophilum* infection.

Five-month-old lambs of the Norwegian White Sheep breed were used in this trial. None of the lambs had previously been on *I. ricinus*-infested pasture, and the lambs were kept indoors during the whole experimental period. Two 16S rRNA variants of *A. phagocytophilum*, i.e., variants 1 and 2 (GenBank accession numbers M73220 and AF336220, respectively), were used as the inoculum (18). Briefly, six lambs were inoculated with various doses of fresh heparinized blood containing the two 16S rRNA variants of *A. phagocytophilum*. Each of two lambs was given an infection dose of variant 1 (approximately  $2.0 \times 10^6$  infected neutrophils [14]) and 1:1,000 (diluted in physiological saline) of that infection dose of variant 2 (group 1), and for another two lambs these doses were reversed (group 2). In addition, each of two lambs was infected with approximately  $2.0 \times 10^6$  infected neutrophils of both variants (group 3). Two

lambs were uninfected controls. All lambs were followed for the next six weeks for clinical observation and blood sampling.

Rectal temperatures were measured daily in all lambs throughout the experimental period, and the clinical manifestations were calculated (22). Blood samples were collected into EDTA daily during the fever period following the inoculation and twice a week after the fever had subsided. From these blood samples, hematological values, including total and differential leukocyte counts, were determined electronically (Technicon H1; Miles Inc.) and blood smears were prepared and stained with May-Grünwald Giemsa.

DNA amplifications were carried out on a PTC-200 instrument (MJ Research). An initial PCR was performed using primers 16S-F5 (5'-AGTTTGATCATGGTTCAGA-3') and ANA-R4B (5'-CGAACAACGCTTGC-3') for amplification of a 507-bp fragment of the 16S rRNA gene in *A. phagocytophilum*. The subsequent seminested reaction with primers 16S-F5 and ANA-R5 (5'-TCCTCTCAGACCAGCTATA-3') produced a 282-bp fragment. PCR products were sequenced directly using Big Dye terminator cycle sequencing chemistry and capillary electrophoresis (ABI 310; Applied Biosystems), and *A. phagocytophilum* variants were detected by visual inspection of the chromatograms (20).

In addition, sera were collected weekly and analyzed using an indirect immunofluorescence antibody assay with *Ehrlichia equi* as an antigen (1, 9, 15).

After inoculation, all lambs reacted with typical signs of *A. phagocytophilum* infection, i.e., high fever ( $>41.0^\circ\text{C}$ ), neutropenia ( $<0.7 \times 10^9$  cells/liter), and cytoplasmic inclusions in neutrophils. The infected lambs developed positive antibody titers to *E. equi* between 7 and 14 days postinoculation. None of these reactions were seen in controls.

No clear differences in clinical manifestations and serologic responses were observed between the lamb groups. The highest number of infected neutrophils in lambs in group 2 (infected with a full dose of variant 2 and 1:1,000 of the infection dose of variant 1) was observed on day 6, while the highest number of infected neutrophils was observed on day 3 in the other two groups.

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TABLE 1. The occurrence of *A. phagocytophilum* 16S rRNA gene variants 1 and 2 in the peripheral blood of three groups of experimentally inoculated lambs<sup>a</sup>

Days after inoculation	Variant(s) detected in:					
	Group 1		Group 2		Group 3	
	Lamb 1	Lamb 2	Lamb 3	Lamb 4	Lamb 5	Lamb 6
0 <sup>b</sup>	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2
2	1	1	2	2	1	1
3	1	1	2	2	1	1
4	1	1	1 and 2	1 and 2	1	1
6	1	1	1	1	1	1
7	1	1	1	1	1	1
8	1	1	1	1	1	1
10	1	1	1	1	1	1
12	1	1	1	1	1	1
14	1	1	1	1	1	1
16		1	1	1	1	1
18		1	1	1	1	2
21	2	2	1	1	1	2
24		2	1			
27						
31					2	
34	1					
37	1	1				1
42						

<sup>a</sup> Each of two lambs was given a full infection dose of variant 1 (GenBank accession no. M73220) and 1:1,000 of that infection dose of variant 2 (GenBank accession no. AF336220) (group 1) and vice versa for another two lambs (group 2). In addition, each of two lambs was infected with a full infection dose of both variants (group 3).

<sup>b</sup> Inoculum only.

Only variant 1 was found by gene sequencing in acutely infected lambs in groups 1 to 3. In group 2, variant 2 was detected on days 2, 3, and 4, while variant 1 was detected from day 4. From day 6 to day 42, variant 1 was detected 53 times, while variant 2 was detected 6 times. Simultaneous infection with both variants was not detected in this period (Table 1).

The two 16S rRNA variants have earlier been used separately in infection studies in lambs. Lambs infected with variant 1 showed more severe clinical manifestations than lambs infected with variant 2. In addition, lambs infected with variant 1 reacted with a positive antibody titer after 14 days, while variant 2-infected lambs were seronegative 4 weeks postinoculation. However, rickettsemias of at least 10 days' duration were observed for both variants during the acute phase of the infection (18).

In the present trial, the double-infected lambs reacted clinically, hematologically, and serologically as though they were infected with variant 1. No clear differences between lamb groups were observed in either clinical manifestations or serologic responses, although the infection rate during the first days of the infection seemed to be lower for group 2 lambs than for the other lamb groups. A delayed onset of clinical symptoms in group 2 may have been caused by a low inoculation dose of variant 1. It has been shown earlier that *A. phagocytophilum* infection in lambs is not dose dependent, except for the incubation period (14).

Infection (superinfection) exclusion is a phenomenon that was first described in bacteriophages (13) but was later confirmed in animal viruses and rickettsiae, such as *Anaplasma marginale* (5, 7). The results of the present study do not sup-

port the existence of infection exclusion in *A. phagocytophilum*-infected lambs but indicate that suppression of genotypes occurs during the acute phase of the infection. This interaction is unidirectional and not dose dependent, as has also been observed when two *ank* gene variants of *A. phagocytophilum* were inoculated simultaneously in BALB/c mice (R. F. Massung, V. E. Pitzer, and M. L. Levin, Abstr. 18th Meet. Am. Soc. Rickettsiol., abstr. 113, 2003). In contrast, when cattle were infected simultaneously with variants of the closely related *A. marginale*, only the variant that was inoculated at the highest dose became established (7). The assumption of infection suppression is also supported by the fact that double infection has been observed only during the acute phase of the infection and not in the persistent phase.

Although both variants were detected in the persistent phase of the infection, one variant seems to dominate. The dominant variant is involved in most fatal cases of tick-borne fever and seems to be the most widespread *A. phagocytophilum* variant in sheep in Norway (17, 18). The reason for this dominance is unknown, but factors such as growth rate, immunogenicity, receptor competition, and antigenic variation may be involved in the infectivity and interaction of *A. phagocytophilum* variants (3, 4, 12).

The prevalence of *A. phagocytophilum* infection in *I. ricinus* ticks in Europe has been found to vary from 1.5 to 34% (6, 21). Natural hosts may therefore be infested with several *A. phagocytophilum*-infected ticks. Simultaneous infection with two variants of the bacterium has been observed earlier in 3 of 40 (7.5%) acutely infected lambs (16). Recently, an acutely infected cow was also found to be infected with two *A. phagocytophilum* variants (20). One should therefore be aware of the possibility that blood in the acute phase of the infection may harbor more than one variant. However, it may not always be possible to detect every variant involved in a single blood sample.

Further studies are in progress to more closely investigate the suppression of genotypes in infected sheep. Studies should also be done with several potential hosts and ticks to analyze the implication of this phenomenon for the epidemiology and control of genotypes of *A. phagocytophilum*, for instance to investigate if individual transmission of single variants occurs, as has recently been assumed for genotypes of *A. marginale* (8).

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