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Identification of *Anaplasma phagocytophila* (Formerly *Ehrlichia phagocytophila*) Variants in Blood from Sheep in Norway

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A total of 41 blood samples were collected from 40 *Anaplasma phagocytophila*-infected sheep in 11 sheep flocks from four different counties of southern Norway. The presence and nature of the *Anaplasma* species were identified by microscopic detection of morulae, PCR, reverse line blot hybridization, and 16S rRNA gene sequencing. *A. phagocytophila* was identified in all of the samples, and sequencing of the 16S rRNA gene revealed the presence of four variants of *A. phagocytophila*. Two of these variants have been described before, but two were newly identified 16S rRNA variants of this species. *A. phagocytophila* variant 1 was found in nine flocks, *A. phagocytophila* variant 2 was found in four flocks, the *A. phagocytophila* prototype was found in two flocks, and *A. phagocytophila* variant 5 was found in one flock. In two flocks, some sheep were infected with *A. phagocytophila* variant 1, whereas others were infected with *A. phagocytophila* variant 2, and in three animals a double infection with two variants was registered. Analyses of the blood samples revealed that blood from sheep infected with *A. phagocytophila* variant 2 contained nearly twice as many neutrophils and eight times as many *Anaplasma*-infected neutrophils as blood from sheep infected with the *A. phagocytophila* variant 1. Furthermore, only 43% of the *A. phagocytophila* variant 2-infected sheep displayed antibody responses in an immune fluorescence assay, whereas 93% of the sheep with the *A. phagocytophila* variant 1-infected sheep were seropositive.

Tick-borne fever (TBF) in sheep caused by *Ehrlichia phagocytophila* and transmitted by the tick *Ixodes ricinus* was the first granulocytic ehrlichial infection to be described and has for decades been a well-known disease in domestic ruminants in several countries in Europe (34). *E. phagocytophila* belongs to the same genogroup as *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent, and natural infection with granulocytic *Ehrlichia* has now been reported in a variety of animal species (9). Recently, Dumler et al. (7) reorganized the families *Rickettsiaceae* and *Anaplasmataceae*, and *E. phagocytophila*, *E. equi*, and the HGE agent were unified into the new species combination *Anaplasma phagocytophila*. For this reason we use *A. phagocytophila* as the emended name for this species throughout this study.

TBF is a common disease in domestic ruminants along the coast of southern Norway (26, 27). In 1995, more than 11,000 sheep flocks were treated prophylactically against TBF with tick repellent and/or insecticides, including ca. 40% of all flocks in Norway (29). In sheep, TBF is characterized by high fever, reduced milk yield, abortion, and reduced fertility in rams. The diagnosis was earlier based on the presence of inclusions (morulae) in circulating neutrophils in Giemsa-stained blood smears (35).

A. phagocytophila infection in sheep is known to produce profound effects on the immunological defense system, which increases susceptiblility to disease and mortality from intercurrent infections such as *Staphylococcus aureus* pyaemia and *Pasteurella haemolytica*/*trehalosi* septicemia (4, 25). Sheep flocks may suffer heavily on *I. ricinus*-infested pastures both due to direct mortality and to impairment of growth rate and production (4). In one flock investigated in Norway, almost one-third of the lambs died on *Ixodes*-infested pastures due to TBF and secondary infections (29). Lamb losses on *I. ricinus*-infested pastures may vary considerably between neighboring farms. The reasons for these variations are unknown but may be caused by differences in virulence between variants of *Anaplasma.* Such variations have earlier been found in both sheep and cattle (8, 31).

The identification of *Anaplasma* and *Ehrlichia* species is difficult because conventional bacteriological methods for cultivation and characterization cannot be used. Morphological and serological methods are also unreliable to differentiate *Anaplasma* and *Ehrlichia* species due to morphological similarities and antigen cross-reactivity between species (22). The purpose of the present study was therefore to identify and compare *Anaplasma* species from sheep with TBF from different areas of Norway by molecular methods. In addition, we wanted to study the number of neutrophils, their infection rate, and the antibody response in infected sheep.

MATERIALS AND METHODS

Animals, blood samples, and hematology. Blood samples were collected from Norwegian sheep with TBF in different *I. ricinus*-infested areas in Norway. A number of collaborating sheep farmers were informed before the tick season, and they were instructed to contact the local veterinarian for blood sampling when a suspected case of TBF was found in their flocks. TBF had earlier caused high mortality in all of these flocks, except in one flock (flock D; see below and Table 3). The rectal temperatures of the actual sheep were measured, and whole

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blood and EDTA-blood samples were collected and sent to the Department of Sheep and Goat Research for further analyses. No further information of the animals was available after blood sampling.

Hematological values, including total and differential leukocyte counts, were determined electronically from the EDTA-blood samples (Technicon H1; Miles, Inc.), and blood smears were prepared and stained with May-Grünwald Giemsa. A total of 400 neutrophils were examined on each smear by microscopy; the numbers of cells containing *Anaplasma* inclusions were recorded, and the percentages of infected neutrophilic granulocytes were calculated. The rest of the EDTA-blood was frozen at −20°C until further analyses could be performed.

Serology. Serum samples were analyzed for the presence of antibodies to *Anaplasma* by an indirect immunofluorescence antibody assay (2). Briefly, twofold dilutions of sera were added to slides precoated with *E. equi* antigen (Protatek, St. Paul, Minn.). Bound antibodies were visualized by fluorescein-isothiocyanate-conjugated rabbit anti-sheep immunoglobulin (Cappel; Organon Teknika, West Chester, Pa.). Sera were screened for antibodies at a dilution of 1:40. If positive, the serum was further diluted and retested. A titer of 1.6 ($\log_{10}$ reciprocal of 1:40) or more was regarded positive.

DNA extraction and PCR amplification. DNA extraction on blood samples was performed according to Olsson Engvall et al. (16), with some modifications. Briefly, 400 μl of thawed EDTA-blood was treated with 220 μl of cold lysis buffer (10 mM Tris-HCl [pH 7.4], 100 mM EDTA), 0.5% sodium dodecyl sulfate, and 10 μl of proteinase K (20 mg/ml) and then mixed gently and incubated at 50°C for 2 h. The mixture was mingled every 15 min, and after 1 h another 6 μl of proteinase K (20 mg/ml) was added. The mixture was then extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with an equal volume of chloroform-isoamyl alcohol (24:1). DNA was precipitated by the addition of a 1/10 volume of 2 M sodium acetate (pH 6.5) and 2.5 volumes of cold ethanol (99%) and was then collected by centrifugation. The pellet was washed once in cold ethanol (70%), dried, and resuspended in 50 μl of sterile water, and the DNA concentration was then measured with a spectrophotometer (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden).

PCR amplifications were performed in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The 5′ part of the 16S rRNA gene of the *Anaplasma* species in the sheep blood samples were amplified in 50-μl volumes consisting of 25 μl of HotStarTaq mix (Qiagen, Hilden, Germany), 4 μl of primer 16S8FE (80 pmol), 4 μl of primer B-GA1B (80 pmol), 2 μl of *tmpB* spike DNA (10 fg) (1), 10 μl of water, and 5 μl of DNA sample. To minimize nonspecific amplification, a touchdown-up PCR program was used: 15 min at 94°C, followed by two cycles of 20 s at 94°C, 30 s at 65°C, and 30 s at 72°C; followed by two cycles under conditions identical to the previous cycles but with an annealing temperature of 63°C. During subsequent two cycle sets, the annealing temperature was lowered by 2°C until it reached 55°C. We then carried out an additional 20 cycles of 20 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by 20 cycles of 20 s at 94°C, 30 s at 63°C, and 30 s at 72°C, followed again by the touchdown program. The PCR was ended by an extra incubation for 7 min at 72°C.

Each time that the PCR was performed, negative (no sample added) and positive (*Anaplasma* or *Ehrlichia* DNA) control samples were included. Each sample was spiked with a critical amount (150 copies) of the *tmpB* spike control DNA to detect any inhibition of the PCR that might lead to false-negative results. When a relatively high concentration of *Anaplasma* DNA was available, the spike was weak or absent. In order to minimize contamination, the reagent setup, the sample addition, and the PCR and sample analysis were performed in three separate rooms, of which the first two rooms were kept at a positive pressure and had airlocks.

Reverse line blot hybridization. The reverse line blotting technique has been described before (11, 12, 21, 23). Briefly, solutions with 5′ amino-linked oligonucleotide probes were coupled covalently to an activated Biodyne C membrane in a line pattern by using a miniblotter (Immunetics, Cambridge, Mass.). After binding of the oligonucleotide probes, the membrane was taken from the miniblotter, washed, and again placed in the miniblotter with the oligonucleotide lines perpendicular to the slots. The slots of the miniblotter were filled with the biotin-labeled denatured PCR product, and hybridization was performed. The membrane was removed from the miniblotter, washed, and subsequently incubated with streptavidin-peroxidase to detect bound biotin-labeled PCR product. After a washing step, hybridization was visualized by incubating the membrane with enhanced chemiluminescence detection liquid (Amersham International, plc., Den Bosch, The Netherlands) and exposing the membrane to X-ray film. For species identification, the biotinylated PCR product was hybridized with 10 different *Anaplasma*- and *Ehrlichia*-specific oligonucleotide probes in the reverse line blot assay. All primers and probes are described in Table 1.

TABLE 1. Oligonucleotide primers and probes used in PCRs and hybridization assays

Oligonucleotide primer or probe	Sequence	Target organism	Target gene	Nucleotide position (range)	Reference or source
Primers					
16S8FE	GGAATTCAGAGTTGGATCMTGGYTCAG	Eubacteria	16S rRNA gene	8–27	L. M. Schouls
B-GA1B	5′biotin-CGGGATCCCGAGTTTGCCGGGACTTCTTCT	*Anaplasma/Ehrlichia* genus	16S rRNA gene	476–456	L. M. Schouls
44k-start	ATGATGTCTATGGCTATAGTCATG	*A. phagocytophila* group	44-kDa gene[a]	49–72	This study
44k-end	ATCTCCCACAACACGATG	*A. phagocytophila* group	44-kDa gene[a]	1180–1197	This study
16SEhrSeq	GGAATTCAGAGTTTGATCMTGGYTCAGAAC	Eubacteria	16S rRNA gene	8–30	This study
GA1BSeq	CGGGATCCCGAGTTTGCCGGGACTTCTTCTGTA	*Anaplasma/Ehrlichia* genus	16S rRNA gene	476–459	This study
Probes					
A-EhrA11	5′ amino-TTATCGCTATTAGATGAGCC	*Anaplasma/Ehrlichia* genus	16S rRNA gene	203–222	L. M. Schouls
A-HGE	5′ amino-GCTATAAAGAATAGTTAGTGG	*A. phagocytophila* prototype	16S rRNA gene	87–107	L. M. Schouls
A-Phago	5′ amino-TTGCTATAAAGAATAATTAGTGG	*A. phagocytophila* variant 1	16S rRNA gene	85–107	L. M. Schouls
A-Phagovar2	5′ amino-GAACGGATTATTCTTTGTAGC	*A. phagocytophila* variant 2	16S rRNA gene	87–106	This study
A-D-Phago	5′ amino-TTGCTATGAAGAATAATTAGTG	*A. phagocytophila* variant 3	16S rRNA gene	87–106	L. M. Schouls
A-D-HGE	5′ amino-GCTATGAAGAATAGTTAGTG	*A. phagocytophila* variant 4	16S rRNA gene	87–106	L. M. Schouls
A-Eschot	5′ amino-GCTGTAGTTTACTATGGGTA	*Ehrlichia* sp., Schotti variant	16S rRNA gene	76–95	L. M. Schouls
A-Ecan	5′ amino-TCTGGCTATAGGAAATTGTTA	*E. canis*	16S rRNA gene	85–105	L. M. Schouls
A-Echaf	5′ amino-ACCTTTGGTTATAAATAATTGTTA	*E. chaffeensis*	16S rRNA gene	83–107	L. M. Schouls
A-EmurisC	5′ amino-GCTATAGGTTCGCTATTAG	*E. muris*	16S rRNA gene	85–103	A. N. Alekseev
A-TmpBEhr	5′ amino-GAAAACTCGAAGAACAAAGAA	*T. pallidum*[b]	*tmpB*	502–522	A. N. Alekseev

[a] Based on the sequence carrying the 44-kDa gene (AF037599) published by Ijdo et al. (10).
[b] That is, *Treponema pallidum*.

TABLE 2. The 5′ end of the 16S rRNA gene sequences (bp 81 to 126) of different *Anaplasma* and *Ehrlichia* strains were determined and compared with similar sequences from GenBank

Sequence[a]	Accession no.	Species	Nucleotide change
TTATTCTTT**A**TAGCTTGCTAT**A**AAGAATA**G**TTAGTGGCAGACGGGTGAGTAATGCA	U02521	*A. phagocytophila* prototype	Prototype
.....**A**.....	M73220	*A. phagocytophila* variant 1	100^{A}
.....**G**.....**A**.....	AF336220	*A. phagocytophila* variant 2	80^{G}, 100^{A}
.....**G**.....**A**.....	AJ242784	*A. phagocytophila* variant 3	92^{G}, 100^{A}
.....**G**.....	AJ242783	*A. phagocytophila* variant 4	92^{G}
.....G.....	AY035312	*A. phagocytophila* variant 5	93^{G}
.....**A**.....G.....	AF227954	*E. phagocytophila*	100^{A}, 121^{G}
.....G.....	AF036647	*E. equi*	90^{G}
.....G.....	AF036646	*E. equi*	94^{G}
.....**GG**..G.....	AF205140	HGE agent	92^{G} 93^{G} 96^{G}

[a] Nucleotides at positions 80, 92, and 100, respectively, are indicated in boldface for each sequence.

DNA sequencing and data analysis. Most PCR products were used directly for sequencing, but some were cloned into a TA-TOPO vector (Invitrogen, Groningen, The Netherlands). The plasmids were isolated and purified by using the Qiagen plasmid minikit and used for sequencing. The PCR products used for DNA sequencing were purified with QiaQuick PCR purification kits (Qiagen). Since PCR products were obtained in a PCR that included a spike control, the PCR yielded a mixture of *Anaplasma* PCR product and the *tmpB* spike. Therefore, we used sequence primers that were specific for the *Anaplasma* PCR product only (16SEhrSeq and GA1BSeq). For DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology was used (Perkin-Elmer, Applied Biosystems Division). The sequenced fragments were separated, and data were collected with an ABI 3700 automated DNA sequencer (ABI, Applied Biosystems Division). The collected sequences were assembled, edited, and analyzed with the DNAStar package (DNAStar, Inc., Madison, Wis.).

Statistics. Statistical calculations on seroprevalences were performed by using the chi-square contingency test, and a two-sample *t* test was used for the hematological variables and the antibody titers (Statistix, version 4.0; Analytical Software). A P value of <0.05 was considered significant.

Nucleotide sequence accession number. The 16S rRNA gene sequences of the new variants of *A. phagocytophila* found in the present study are available in the GenBank database under the accession numbers AF336220 and AY035312.

RESULTS

Blood samples. Altogether 41 blood samples from 40 different sheep were collected. Two samples originated from the same sheep and were drawn 1 month apart. All sampled sheep revealed clinical signs of TBF, such as fever, increased respiration, dullness, and inappetence. Concurrent diseases were not observed. The samples were from 11 sheep flocks in four different counties of southern Norway and were collected from April to October in two consecutive years. The age of the sheep varied from 1 month to 2 years; however, most of the animals (68%) were less than 4 months old.

Reverse blot line hybridization and DNA sequence analysis. In order to confirm the results observed by the reverse line blot assay, the PCR products used in hybridizations were also sequenced. Although the sequence analysis largely confirmed the reverse line blot results, additional sequence variation was found. The blood samples carried *A. phagocytophila* that displayed minor sequence variation of the 16S rRNA gene and were designated variants. Two samples carried 16S rRNA gene sequences identical to the *A. phagocytophila* prototype (GenBank accession no. U02521) and a second group carried 16S sequences identical to the sequence with the accession number M73220 that differed at nucleotide position 80 from the prototype sequence and was designated variant 1. The largest group of sheep carried *A. phagocytophila* that differed at positions 80 and 100 of the 16S rRNA gene, and this type was designated *A. phagocytophila* variant 2 (accession no. AF336220). One sample contained the new *A. phagocytophila* variant 5, which differed at position 93 of the 16S gene (accession no. AY035312). The prototype and all variant signature sequences, including some other published sequences, are displayed in Table 2.

A probe was designed to detect the *A. phagocytophila* variant 2, and all samples were retested on a reverse line blot that included this probe (Fig. 1). This analysis was in complete concordance with the sequence analysis and confirmed that the observed sequence variation was not an artifact introduced by the sequencing procedure.

When blood samples from a total of 11 sheep flocks were examined, *A. phagocytophila* variant 1 was found in nine flocks, *A. phagocytophila* variant 2 was found in four flocks, *A. phagocytophila prototype* was found in two flocks, and *A. phagocytophila* variant 5 was found in one flock. In two flocks, some sheep were infected with *A. phagocytophila* variant 1, whereas others were infected with *A. phagocytophila* variant 2. In three animals the PCR product reacted with two different *Anaplasma* probes in the reverse line blot, which might indicate a double infection with two different variants. DNA sequencing of these PCR products revealed ambiguous bases at a few positions in the sequence, supporting the supposition that double infection with two variants had occurred. For this reason the PCR products were cloned into a plasmid, and the inserts of 10 clones of each cloned PCR product were sequenced. This indeed revealed the simultaneous presence of two different variants in these three animals. Both samples of the one animal that was sampled twice carried the same variant of *A. phagocytophila*. In one flock, where 21 animals were examined, 3 (14%) were infected with *A. phagocytophila* variant 1, and 18 (86%) were infected with *A. phagocytophila* variant 2 (Table 3).

To exclude the possibility that *A. phagocytophila* carried two different copies of the 16S rRNA gene, we performed a Southern blot hybridization with a biotin-labeled 16S rRNA oligonucleotide probe on *Xba*I- and *Pst*I-digested genomic *A. phagocytophila* DNA. This revealed the presence of a single 16S rRNA gene in the genome (data not shown). This result was not completely unexpected since BLAST searches in the *E. chaffeensis* genome sequence had also shown that this *Ehrlichia* species contains a single 16S rRNA gene.

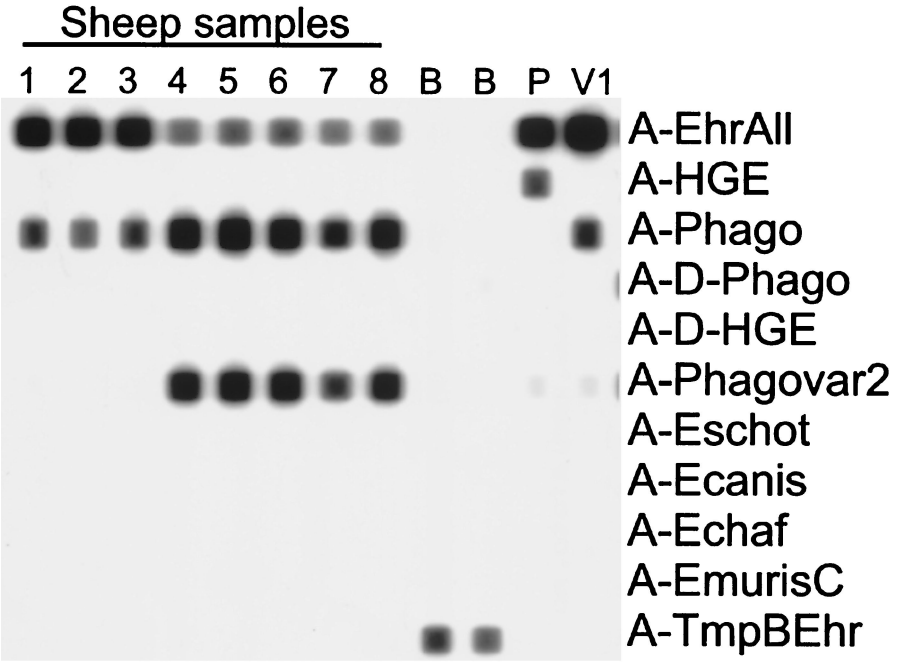

FIG. 1. Reverse blot analysis of PCR products obtained from blood samples of *A. phagocytophila*-infected sheep. The oligonucleotide probes are shown as lines in the horizontal direction, and the biotin-labeled PCR products are perpendicular in the vertical direction. Samples 1 to 3, samples from *A. phagocytophila* variant 1-infected sheep; samples 4 to 8, samples from *A. phagocytophila* variant 2-infected sheep; B, blank controls (no DNA added); P, *A. phagocytophila* prototype-positive PCR control; V1, *A. phagocytophila* variant 1-positive PCR control.

Clinical parameters, hematology, and serology. Clinical variables at the time of blood sampling were obtained from 37 sheep. A marked and significant difference was found in the number of neutrophils. Blood samples from sheep infected with *A. phagocytophila* variant 2 contained nearly twice as many neutrophils as blood samples from sheep infected with *A. phagocytophila* variant 1. In addition, blood from *A. phagocytophila* variant 2-infected sheep carried eight times as many neutrophils with *Anaplasma* inclusions as blood from sheep infected with *A. phagocytophila* variant 1. The clinical parameters and hematology in sheep infected with different variants of granulocytic *Anaplasma* are shown in Table 4.

Antibody titers to *E. equi* measured at the day of blood sampling are shown in Table 4. Only 24 of 39 (62%) of the acute *Anaplasma*-infected animals were found to be seropositive at the time of sampling. Remarkably, 93% of the *A. phagocytophila* variant 1-infected animals carried antibodies reacting with the *E. equi* antigen, whereas only 43% of the sheep infected with *A. phagocytophila* variant 2 were seropositive ($P < 0.02$). However, the mean antibody titer ($\log_{10}$) was not significantly different between sheep in these two variant groups.

TABLE 3. *A. phagocytophila* variants from 11 sheep flocks in Norway identified by reverse line blot hybridization and DNA sequencing

Flock no.	No. of sheep	No. of sheep positive for:			
		Variant 1	Variant 2	Prototype	Variant 5
A	2	2			
B	1	1			
C	3[a]	3		1	
D	21	3	18		
E	2	2			
F	1		1		
G	2	1	1		
H	2	2			
I	1[a]	1			1
J	4[a]	4		1	
K	1		1		
Total	40	19	21	2	1

[a] One sheep infected with two variants of *A. phagocytophila*.

DISCUSSION

We found four 16S rRNA gene sequence variants of *A. phagocytophila* in blood from sheep suffering from TBF. To our knowledge, three of these variants have not earlier been identified in sheep, and two of them have not been identified in any other study before. Nucleotide differences at 16S rRNA level in *A. phagocytophila* have been found in isolates from rodents, deer, and *Ixodes* ticks (3, 5, 6, 15, 17, 20, 23, 32, 33). However, whether all variants can cause disease in humans and animals remains to be determined. Therefore, the importance of these sequence differences remains to be elucidated. In the study presented here, at least two of the variants found seem to cause classical *A. phagocytophila* infection in sheep.

The sampled sheep were more than 1 month old. Age resistance in lambs older than 1 month and variation in clinical symptoms among Norwegian sheep breeds have not been found in experimentally *A. phagocytophila*-infected lambs (24).

TABLE 4. Clinical variables (geometric mean ± SD) and antibody titer to *E. equi* antigen in 38 sheep infected with different variants of *A. phagocytophila*

A. phagocytophila variant(s)	*n*	Result (mean ± SD)				
		Rectal temp (°C)	No. of neutrophils (10^9 cells/liter)[a]	Absolute no. of infected neutrophils (10^9 cells/liter)[a]	No. of seropositive sheep[b] (%)	Mean antibody titer in seropositive sheep
A. phagocytophila variant 1	14[c]	40.74 ± 1.015	0.80 ± 0.182	0.024 ± 0.004 (3.1 ± 2.91)	13 (93)	1:386
A. phagocytophila variant 2	21	40.64 ± 1.014	1.48 ± 0.227	0.193 ± 0.006 (13.1 ± 3.07)	9 (43)	1:320
A. phagocytophila prototype and variant 1	2	41.54 ± 1.005	1.38 ± 1.154	0.067 ± 0.013 (4.6 ± 1.53)	2 (67)	1:452
A. phagocytophila variant 1 and variant 5	1	40.40	1.62	0.008 (0.5)	0	Negative

[a] The number of neutrophils and infected neutrophils in sheep infected with *A. phagocytophila* variant 1 and those infected with *A. phagocytophila* variant 2 were significantly different: $P < 0.03$ and $P < 0.004$, respectively. Percent values are given in parentheses.
[b] Comparison of *A. phagocytophila* variant 1 and *A. phagocytophila* variant 2: Yates corrected $\chi^2 = 6.98$ and $P < 0.01$.
[c] Clinical data from one of the *A. phagocytophila* variant 1-infected sheep were not available.

In the present study, the number of neutrophils, the number of infected neutrophils, and the serological response differed significantly between sheep infected with either *A. phagocytophila* variant 1 or 2. In the flock with few disease problems, 86% of the variants were of the *A. phagocytophila* variant 2 type. One possible reason for this difference could be that the *A. phagocytophila* variant 2 is better equipped to elude the immune system by inhibiting antibody response, resulting in more proliferation within granulocytes. This theory is supported by the observation in mice that pathology due to host immunity seems to play a more important role than pathogenicity of *Anaplasma* itself during infection with HGE (14). However, the role of host immunity in the pathogenesis of TBF in sheep has to be elucidated. Alternatively, the differences in morbidity and antibody response may be explained by sampling in the later phase of the infection in case of *A. phagocytophila* variant 1-infected sheep. However, later sampling may also have been caused by less-apparent acute disease manifestations in the *A. phagocytophila* variant 2-infected animals. The time point of sampling is important since earlier studies have shown that, in the later phase of the acute infection, both the number of neutrophils and the rate of infected neutrophils decrease (35).

Different clinical and serological responses between variants of *Anaplasma* have earlier been observed in experimental infections in cattle, horses, and sheep (8, 19, 28, 31). In the present study it was difficult to compare different clinical and serological values since only single point measurements were available. However, a recent experimental inoculation study in lambs of a single breed revealed a significant difference in the clinical, hematological, and serological responses between these two variants of *A. phagocytophila* (S. Stuen, K. Bergström, M. Petrovec, I. Van De Pol, and L. M. Schouls, unpublished data).

The present serology results indicate that only 61% of the acute *Anaplasma*-infected animals were seropositive at the time of sampling. This result is in accordance with an earlier study in which 22 of 30 (73%) of *Anaplasma*-positive animals were found to be seropositive (16).

An earlier experimental needle inoculation trial in sheep with *A. phagocytophila*-infected blood indicated that infected neutrophils may be found by Giemsa-stained blood smears examination several days before seroconversion appears (18). In addition, some *Anaplasma*-infected lambs have been found to remain seronegative for up to 6 weeks after the primary inoculation with a species similar to the HGE agent (28). Serologic investigation is therefore not reliable as the only diagnostic tool to detect acute *Anaplasma* infection in sheep.

There has been much debate about the species definition and nomenclature of the group of granulocytic *Ehrlichia*. Recently, Dumler et al. (7) clarified this by unifying *E. phagocytophila*, *E. equi*, and the HGE agent into a single species: *A. phagocytophila*. However, there are minor differences in the 16S rRNA gene of the latter species, and these differences can be used to differentiate particular groups within the species *A. phagocytophila*. Typing within species will require more polymorphism than the limited variation found in the 16S rRNA gene. Therefore, analysis of particular highly polymorphic sequences or of a number of housekeeping genes, such as those used in the multilocus sequence typing, are required for reliable discrimination of strain types. Variations in other genes, especially those coding for surface proteins, are more likely to affect properties such as virulence, host range, and interaction with arthropod vectors. Recently, it has been shown that sera from mice with high concentrations of antibodies that bind to the P44 proteins of the HGE agent or monoclonal antibodies specific to these proteins partially protect mice from the infection (13, 30) and that this protein may be located in the outer membrane of the HGE agent (36). This suggests that the P44-protein specific antibodies may play a role in the immunity against this infection and that the genes encoding the P44 outer membrane proteins may have a role in pathogenesis and immunresponse in *A. phagocytophila* infection in mice (10, 13, 37).

In conclusion, the present study shows the existence of different *A. phagocytophila* variants in sheep: within different flocks, within each flock, and also within a single animal. Variants of *A. phagocytophila* causing TBF in sheep may accordingly exist simultaneously on the same pasture and may cause differences in both clinical and immunosuppressive reactions within each flock. However, the clinical and epidemiological consequences of these findings have to be further elucidated.

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