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# Dynamic Transmission of Numerous *Anaplasma phagocytophilum* Genotypes among Lambs in an Infected Sheep Flock in an Area of Anaplasmosis Endemicity▽

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**The transmission dynamics of *Anaplasma phagocytophilum* strains circulating within juvenile members of a sheep flock grazing on an *Ixodes ricinus*-infested pasture in southern Norway were monitored. PCR-based detection of the bacterial *p44* fragments in the blood of 16 lambs sampled weekly for 16 weeks following their release into pasture revealed rickettsemia in all animals, with an increasing proportion of infected animals as the survey progressed. Comparison of partial *msp4* sequences obtained from infected blood samples revealed 24 distinct genotypes, some of which were repeatedly encountered, occurring in up to six sheep over a 14-week period, whereas others were observed only once. Individual sheep were infected by up to five distinct genotypes, with a specific genotype being encountered for between one and three consecutive weeks, and in some sheep, genotypes detected early in the study were also present in later samples. In general, detection of *A. phagocytophilum* by PCR correlated well with the observation of infected neutrophils in blood smears. Together these results reveal a previously unrecognized diversity of *A. phagocytophilum* strains simultaneously circulating within an infected population in an area of endemicity and are consistent with a remarkably dynamic transmission of strains among infected animals.**

*Anaplasma phagocytophilum* is an ixodid tick-transmitted bacterial parasite of mammals that is widely encountered across the temperate latitudes of the Northern Hemisphere. *A. phagocytophilum* has long been recognized as the agent of tick-borne fever in European sheep flocks, and the disease remains of veterinary significance today. Indeed, the economic implications of the morbidity and mortality associated with tick-borne fever for the modern livestock farming industry are well established (5). The ability of *A. phagocytophilum* to cause disease in other hosts, including humans, has recently become apparent, particularly with the emergence of human granulocytic anaplasmosis, a syndrome that is now one of the most frequently encountered human tick-borne infections (9).

In Europe, the exophilic tick species *Ixodes ricinus*, which has a broad host range, is considered the most important vector of *A. phagocytophilum*. However, other nidiculous *Ixodes* species, which have a far more limited host range, such as *Ixodes trianguliceps*, have also been proposed and/or demonstrated to be competent vectors (2, 3). Although *A. phagocytophilum* is transstadially maintained in infected ticks, transovarial transmission does not occur (15, 20, 22); hence, exploitation of mammalian reservoir hosts is essential to its natural persistence.

Studies over the past few years have revealed that *A. phagocytophilum* has a wide mammalian host range, with infections being detected in several species of rodents, domesticated and wild-living ruminants, companion animal species, and humans (1, 6, 13, 15, 20, 25, 34). Among these, rodents and ruminants, which are key hosts for vector ticks, are thought to serve central roles in the maintenance of *A. phagocytophilum* in nature as important reservoir hosts; surveys of populations of species belonging to both groups of animals have revealed that a significant proportion are subclinically infected with the bacterium (1, 10, 34, 35). The role of sheep in the natural maintenance of *A. phagocytophilum* has been studied in some detail, and it is clear that in some settings they serve as its main reservoir. For instance, Ogden and colleagues (21, 22) demonstrated the maintenance of *A. phagocytophilum* in "fell"-grazing sheep flocks in the absence of other important hosts such as rodents or deer. Early studies revealed that *A. phagocytophilum* infections can persist for several months or longer in sheep (15), and more recent experimental studies have further quantified their dynamics (11, 31).

Comparative analysis of 16S rRNA-encoding gene sequences has been widely used to delineate among *A. phagocytophilum* strains, although only a few genotypes have been encountered (32). Investigation of other loci such as *ankA* and *msp4* has revealed far greater intraspecies sequence variation and the existence of numerous distinct genotypes (4, 8, 26, 36). The second of these two loci, *msp4*, encodes a putative outer membrane protein that shares a high degree of sequence similarity with *Anaplasma marginale* MSP4, an immunodominant surface protein of unknown function (19). The sequence sta-

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bility of *msp4* during the in vitro passage of *A. phagocytophilum* has been demonstrated (4).

The biological implications of *A. phagocytophilum* strain diversity have yet to be elucidated; although some associations have been proposed (16), no consistent correlations between genotypic delineations and geographical and/or host provenance have been reported. A previous study, in areas of Norway where pasture fever is endemic, identified 16S rRNA gene fragment sequence variants of *A. phagocytophilum* within several sheep flocks and occasionally in the same animal (32), revealing that multiple strains were concurrently circulating within the flock. However, as yet, no efforts to examine the infection dynamics of coexisting *A. phagocytophilum* strains in sheep or other reservoir hosts have been reported.

In this study we have used a variety of approaches to examine the infection dynamics and diversity of *A. phagocytophilum* strains circulating in lambs within a naturally infected sheep flock. Our findings have important consequences for our understanding of the epidemiology of *A. phagocytophilum* enzootic infections.

## MATERIALS AND METHODS

**Management of sheep and blood collection.** The sheep that were monitored for this study were part of a flock reared on a farm near Sandnes in southern Norway, which had a long history of tick-borne fever in its stock. The flock was held indoors throughout winter and during spring until lambing was completed. On 1 May 2006 the flock, comprising 60 ewes and 90 lambs, was treated with synthetic pyrethroid (Coopersect Vet; Schering-Plough), and on 7 May, 16 lambs were chosen at random for inclusion in the study. Each lamb was examined for clinical abnormalities and tick infestation on the head, its rectal temperature was recorded, and a blood sample was collected from the jugular vein. On 8 May the flock was turned out onto a 500-ha fenced pasture, where the sheep remained for the duration of the study period. In line with normal farming practice in the area, pyrethroid treatment was repeated on days 21 and 56 of the study (25 May and 27 June). The 16 lambs, identified by ear tags, were captured each week for the next 16 weeks (finishing 23 August). On capture, each lamb was examined as described above and a blood sample was collected. Sera and whole-blood samples (in EDTA) were stored at −20°C until required. A blood smear, made at the time of blood collection, was fixed in methanol and then stored at room temperature.

**Nucleic acid extraction and PCR amplifications.** Nucleic acid extracts were prepared from whole-blood samples as previously described (14), with one sterile sheep blood control being concurrently processed with every four study samples. Extracts were then stored at −20°C until required. The presence of *A. phagocytophilum* DNA in nucleic acid extracts was initially determined using a real-time PCR assay targeting an *msp2*/*p44* fragment, as previously described (3, 7). Samples yielding a positive result using this assay were retested for confirmation. For genotyping, each nucleic acid extract in which *A. phagocytophilum* DNA was detected using the assay described above was incorporated into a second PCR assay targeting an *msp4* fragment, as previously described (4). Some nucleic acid extracts were also incorporated into a third PCR assay targeting a 16S rRNA gene fragment, as previously described (17). Each stage of all PCR assays was performed in separate rooms within a suite specifically designed for the purpose; thus, nucleic acid extractions and the preparation of PCR mixes were physically separated from amplification and subsequent steps. In addition to the cross-contamination controls discussed above, each PCR incorporated positive and negative assay controls.

**DNA sequencing and sequence analysis.** Partial *msp4* and 16S rRNA gene amplification products were purified (QIAquick; Qiagen), and then the sequence of nucleic acids on both strands was determined by a commercial sequencing service using reactions incorporating the same primers as those used in the relevant PCR amplifications. The products of sequence reactions were visualized and verified; then, for each amplification product, the results of each strand were combined using ChromasPro (Technelysium Pty Ltd). These sequences were then edited by the removal of primer sequences on either extremity prior to comparison using Align Plus (Scientific and Educational Software Ltd).

**Determination of in vivo *msp4* sequence stability.** *msp4* fragments were amplified from DNA extracts prepared from blood samples drawn from three experimentally infected lambs held in solitary, tick-free confinement. Two of the lambs were infected with the AF336220 strain and one with the M73220 strain (28). Blood samples were collected from each lamb soon after inoculation (day 5 or 7 postinoculation) and several weeks later (day 42, 48, or 93 postinoculation). Amplification products were sequenced, and the *msp4* sequences obtained from each blood sample were compared with each other as described above.

**Examination of blood smears and determination of serum antibody titers.** Fixed blood smears were stained using May-Grünwald Giemsa stain and then microscopically examined (1,000× magnification) for the presence of intraneutrophil morulae consistent with *A. phagocytophilum* infection. A total of 400 neutrophils were examined in each smear (31). The presence of anti-*A. phagocytophilum* antibodies in serum samples was determined and quantified using a previously described immunofluorescence antibody test (IFAT) (27).

**Nucleotide sequence accession numbers.** The 24 partial *msp4* sequence types have been submitted to GenBank and have been allocated the following accession numbers: 6018-05-07 (*msp4* sequence type A), EU240465; 6018-02-08 (*msp4* sequence type B), EU240466; 6018-16-08 (*msp4* sequence type C), EU240467; 6018-23-08 (*msp4* sequence type D), EU240468; 6022-16-05 (*msp4* sequence type E), EU240469; 6019-09-08 (*msp4* sequence type F), EU240470; 6020-27-06 (*msp4* sequence type G), EU240471; 6021-21-06 (*msp4* sequence type H), EU240472; 6021-02-08 (*msp4* sequence type I), EU240473; 6021-16-08 (*msp4* sequence type J), EU240474; 6022-05-07 (*msp4* sequence type K), EU240475; 6022-23-08 (*msp4* sequence type L), EU240476; 6023-05-07 (*msp4* sequence type M), EU240477; 6023-16-08 (*msp4* sequence type N), EU240478; 6025-09-08 (*msp4* sequence type O), EU240479; 6025-16-08 (*msp4* sequence type P), EU240480; 6026-27-08 (*msp4* sequence type Q), EU240481 6028-09-08 (*msp4* sequence type R), EU240482 6028-23-08 (*msp4* sequence type S), EU240483; 6029-27-06 (*msp4* sequence type T), EU240484; 6032-09-08 (*msp4* sequence type U), EU240485; 6036-16-08 (*msp4* sequence type V), EU240486; 6046-09-08 (*msp4* sequence type W), EU240487; 6046-23-08 (*msp4* sequence type X), EU240488.

## RESULTS

**Herd health, clinical signs, and tick infestation.** During the study period, the flock remained generally healthy, although one lamb died of tick-borne fever and another was lost on pasture. None of the 16 lambs that were monitored developed any symptomatic illness, with clinical evaluation prior to each blood collection revealing few abnormalities. Signs of depression were noted in some lambs with rectal temperatures of >41°C (see Table 1). One lamb (6032) developed a subcutaneous abscess during June, and this was opened on 12 July and subsequently resolved. No antibiotics were prescribed to any lambs during the study period. *Ixodes ricinus* ticks were often noticed on the heads of the lambs, but these were not quantified.

**Rickettsemia in the study animals.** The real-time PCR assay revealed the presence of *A. phagocytophilum* DNA in 105 blood samples, including at least two from each of the 16 study animals (Table 1). Two lambs were rickettsemic within 14 days of their release, and from day 28 the number of rickettsemic lambs gradually increased until day 63, when *A. phagocytophilum* was detected in the blood of 11 animals. After a dip between days 64 and 71, the prevalence of rickettsemia began to rise again to a peak of 15 of 16 lambs toward the end of the study (Table 1). The number of infected blood samples collected from each animal varied, ranging from 2 (lamb 6020) to 12 (lamb 6046). Of the 105 real-time PCR-positive blood samples, *A. phagocytophilum* DNA was also detected in 85 using the PCR targeting an *msp4* fragment. Furthermore, infected neutrophils were observed by examination of blood smears in 65 samples. All 85 *msp4* PCR-positive samples and all 65 blood smear-positive samples were also positive by real-time PCR. All but one of the 65 blood smear-positive samples were also positive by *msp4* PCR (Table 1).

**Confirmation of *msp4* sequence stability in vivo.** In all three sheep studied, the *msp4* sequence obtained early during the course of experimental infection was indistinguishable from that obtained several weeks later. For the first sheep, samples were collected 88 days apart, for the second, 37 days apart, and for the third, 41 days apart. The *msp4* sequences obtained from the lambs infected with the same *A. phagocytophilum* strain were indistinguishable from one another.

**Genetic variation among infecting *A. phagocytophilum* strains.** The nucleotide base sequences of all 85 products obtained from the PCR targeting an *msp4* fragment were successfully determined and were compared with one another. A total of 24 different sequence types were encountered (Table 1). Comparison of these sequence types with those previously reported for *A. phagocytophilum* revealed that 21 had not previously been encountered. The three exceptions matched partial *msp4* sequences obtained from livestock in the United Kingdom and bison in Poland. Alignment of *msp4* sequences revealed that all were similar to one another, with substitutions at only 23 of 301 base pairs analyzed. The two most dissimilar sequences shared 95% identity. All but one of the substitutions observed occurred in the third base position of a codon and were synonymous. The A/G polymorphism at base pair 41 was nonsynonymous, resulting in isoleucine/valine amino acid variation. Phylogenetic inference of evolutionary relationships among the 21 new *A. phagocytophilum msp4* sequence types and those previously determined indicated the specific clustering of all the newly encountered strains with all those previously except for the *A. phagocytophilum* roe deer strain, a known outlier within the genetic diversity of the species (8) (data not shown). However, within this cluster, the branching orders proposed by different analytical methods generally varied, with very few being conserved and/or supported by high bootstrap scores.

Partial 16S rRNA-encoding gene sequences were obtained from eight of the nucleic acid extracts that yielded partial *msp4* sequence type A (Table 1). Comparison of these 16S rRNA gene sequences revealed them all to be indistinguishable from one another and from a sequence previously encountered among *A. phagocytophilum* strains infecting Norwegian sheep (referred to as 16S rRNA variant 1) (32). This 16S rRNA variant was also obtained from representatives of *msp4* sequence types D, F, G, J, K, and L (Table 1). The 16S rRNA variant 5 (also previously encountered among Norwegian sheep) was obtained from a representative of *msp4* sequence type T (Table 1), but a previously unreported 16S rRNA gene variant (named variant 6) was obtained from a representative of *msp4* sequence type C (Table 1). The sequences for variants 1 (ATAAAGAATAA), 5 (ATGAAGAATAG), and 6 (TTGA AGAATAA) were very similar, varying at only three base positions. (Nucleotide positions are numbered 90 to 100 in the 16S rRNA gene sequence of the *A. phagocytophilum* prototype [accession no. U02521].)

**Transmission dynamics of *A. phagocytophilum* strains.** As described above, 24 *msp4* sequence types were identified among the strains circulating within the study flock during the sampling period. On only 4 of the 85 occasions on which *msp4* sequences were obtained for a PCR-positive sample was evidence of coinfection by multiple *A. phagocytophilum* strains obtained (Table 1).

TABLE 1. Longitudinal survey of *A. phagocytophilum* genotypes infecting the blood of 16 Norwegian lambs[a]

| Lamb no. | Result(s) for sample collected at day (date) post-release onto tick-infested pasture | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 (9 May) | 14 (16 May) | 21 (25 May) | 28 (31 May) | 35 (7 June) | 42 (14 June) | 49 (21 June) | 56 (27 June) | 63 (5 July) | 70 (12 July) | 77 (19 July) | 84 (26 July) | 91 (2 August) | 98 (9 August) | 105 (16 August) | 112 (23 August) |
| 6018 | | | | | | | | + | A [8] (1) | A [10] (1) | | | B [1] | | C [50] | D [3] |
| 6019 | | A [22] (1) | MIX [1] | A [1] (1) | | | A [1] | | | | A [1] (1) | | + | F | D [1] | |
| 6020 | | | | | | | | G [24] (1) | | | | G [1] | | | | |
| 6021 | | | | | | | H | | | | | | I [40] | I [3] | J [3] | J [5] |
| 6022 | | E | + | | | | + | | K [3] (1) | + | F [3] (1) | F | | C [1] | C [1] | L [2] |
| 6023 | | | | | | + | | | M | | | | | | N [2] | |
| 6024 | | | | | | | | **F [45]** | + | | | | + | **I [32]** | + | C [27] |
| 6025 | | | | | | | | | | | | | | O [5] | P [9] | L [10] |
| 6026 | | | | | | | | Q | | | | | | **MIX [41]** | O [2] | D [10] |
| 6028 | | | | | | **D [69] (1)** | D [1] | A [2] (1) | A [2] (1) | + | | A [1] | | R | P | S [6] |
| 6029 | | | | | | H | | T [1] (5) | T [3] | + | | T | + | O | + | **A [51]** |
| 6031 | | | | | | **L [21] (1)** | [1] | | N [4] | | | L [1] | | | R [2] | P |
| 6032 | | | | | C [1] (6) | C [1] | C | A [2] | A [1] | C [23] | | C | A | U [1] | U [2] | A [1] |
| 6036 | | | | | E [1] | | | | E [1] | | | | | | V [57] | MIX |
| 6045 | | | | | | | | | **J [45] (1)** | J | | | + | + | E | MIX [1] |
| 6046 | | | U [1] | U | U [1] | | **A [2] (1)** | A [1] | + | U [6] | + | | + | W | O | X [13] |

[a] Plus signs indicate blood samples that yielded only a *p44* real-time PCR amplification product, whereas letters indicate samples that also yielded an *msp4* PCR amplification product. Letters A to X represent the *msp4* sequence type detected. Brackets indicate blood smears in which *A. phagocytophilum* infections were observed, and the number within the brackets is an estimate of the percentage of neutrophils infected with *A. phagocytophilum*; blood smears were prepared every time a blood sample was collected, but only those found to be infected are indicated in the table. Parentheses indicate that a partial 16S rRNA gene sequence was obtained for that sample, and the number within the parentheses is the sequence type encountered. Boldface indicates that the temperature of the lamb was abnormally high (>41°C) when the blood sample was collected.

TABLE 2. Details of IFAT results

| Lamb no. | Date of birth (day/mo/yr) | Ewe no. | *A. phagocytophilum* IFAT titer for sample collected on date (day/mo/yr) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 9/5/06 | 25/5/06 | 21/6/06 | 19/7/06 | 23/8/06 |
| 6018 | 16/4/06 | 1 | <40 | 320 | <40 | 2,530 | 5,120 |
| 6019 | 16/4/06 | 1 | 1,280 | 1,280 | 320 | 80 | 5,120 |
| 6020 | 16/4/06 | 1 | 320 | 80 | <40 | <40 | <40 |
| 6021 | 18/4/06 | 2 | 640 | 80 | <40 | 160 | 2,560 |
| 6022 | 18/4/06 | 2 | 640 | 80 | <40 | 320 | 5,120 |
| 6023 | 21/4/06 | 3 | 320 | 160 | <40 | 640 | 1,280 |
| 6024 | 21/4/06 | 3 | 160 | 80 | <40 | 2,560 | 10,240 |
| 6025 | 20/4/06 | 4 | 1,280 | 640 | 320 | 80 | 640 |
| 6026 | 20/4/06 | 4 | 1,280 | 320 | 160 | <40 | 320 |
| 6028 | 21/4/06 | 5 | 320 | 160 | 1,280 | 1,280 | 5,120 |
| 6029 | 21/4/06 | 5 | 640 | 160 | 40 | 2,560 | 5,120 |
| 6031 | 22/4/06 | 6 | 1,280 | 160 | 1,280 | 2,560 | 10,240 |
| 6032 | 22/4/06 | 6 | 640 | 160 | 1,280 | 5,120 | 10,240 |
| 6036 | 25/4/06 | 7 | 640 | 80 | 320 | 320 | 1,280 |
| 6045 | 27/4/06 | 8 | 320 | 160 | <40 | 20,480 | 5,120 |
| 6046 | 27/4/06 | 8 | 640 | 320 | 2,560 | 10,240 | 20,480 |

Although some *msp4* sequence types were encountered over two or three consecutive weeks, a far more frequent observation was that whenever a specific sequence type was detected it was no longer present the following week (Table 1). All but one animal were infected with multiple sequence types during the course of our study, with up to five different *msp4* sequence types being detected in some animals (Table 1). Some sequence types were reencountered in an individual several weeks subsequent to their initial detection; in some instances the samples collected between initial and subsequent detection of a specific sequence type contained other sequence types (e.g., lamb 6032, days 35 to 70 [Table 1]), whereas in others, no rickettsemia could be detected (e.g., lamb 6019, days 28 to 77 [Table 1]).

Some of the *msp4* sequence types were encountered more frequently than others (Table 1). Sequence type A was the most frequently encountered sequence type, being detected in 16 blood samples from six different lambs, whereas eight sequence types were encountered only once. As most *msp4* sequence types were encountered on only one or two occasions, we did not attempt to test whether there were statistically significant differences in the nature (length, intensity, and periodicity) of the rickettsemia induced by different sequence types. The intensities of rickettsemias varied markedly among samples, occasionally rising to a level where over half the circulating neutrophils were parasitized (Table 1). However, in the majority of infected smears examined, a far lower percentage (1 to 2%) of neutrophils contained morulae (Table 1).

**Antibody responses to *A. phagocytophilum* infection.** All lambs except 6020 developed detectable anti-*A. phagocytophilum* antibodies during the study period (Table 2). However, the general pattern of antibody titers was one in which initially high titers gradually subsided by mid-June, only to reappear or increase by mid-August (Table 2).

## DISCUSSION

This study is the first to apply sensitive detection and genotyping methods to investigate the presence and diversity of *A. phagocytophilum* strains infecting a single flock of sheep and, through repeated sampling of the same animals, to follow the longitudinal dynamics of rickettsemias caused by specific genotypes. Perhaps the most unexpected finding of this study was the presence of 24 distinct *A. phagocytophilum* genotypes circulating in the flock. Although this degree of diversity is previously unrecognized, our findings are consistent with earlier work in which 16S rRNA variants have been concurrently encountered within the same flock (32). Characterization and delineation of *A. phagocytophilum* genotypes were primarily based on comparison of partial *msp4* sequences, an established approach that has been shown to have clear benefits in terms of practicality and sensitivity (4, 8). Furthermore, through the study of *A. phagocytophilum* strains in experimentally infected animals we have demonstrated that *msp4* sequence type remains unchanged for at least 88 days in vivo. This observation adds to previous in vitro work (4) in substantiating the stability of the locus and hence the validity of *msp4* sequence comparison as a reliable approach for *A. phagocytophilum* genotyping. Although the *msp4* sequence types encountered were very similar to one another, that each is a truly distinct genotype is supported by several lines of evidence. Firstly, at 18 of the 23 polymorphic sites within an alignment of the *msp4* sequence types that we obtained, each sequence variant was shared by more than one sequence type. Furthermore, for two of the five sequence types that possessed a unique point mutation, multiple examples of that sequence type were encountered (J and T). For the remaining three sequence types (M, W, and X), which were encountered only once, repeated *msp4* amplification and sequencing yielded the same polymorphisms. In addition to this, all but one polymorphism were synonymous and none resulted in either frameshifts or the creation of a stop codon. That 21 of the 24 *msp4* sequence variants encountered in this study had not been reported previously (4, 8) suggests that our knowledge of the diversity of this locus appears to be very much in its infancy.

The direct sequencing of *msp4* amplicons does not lend itself well to detecting coinfections, particularly when one sequence type numerically dominates the amplicon pool. We therefore cannot rule out the possibility that coinfections by different *msp4* sequence types were far more common than our observations suggested. Nonetheless, although we considered that the approach that we used merely identified the major contributing sequence type of a potential guild of infecting *A. phagocytophilum* strains, the clarity of most of the sequence data obtained indicated that the characterized sequence type was the overwhelming contributor to the guild and therefore probably the most important in terms of transmission. Our observation of a single genotype dominating the community of *A. phagocytophilum* strains in the blood is in keeping with previous studies (29, 33). However, our results also suggest that a dominant genotype does not remain so for long.

The frequent temporal changes in the dominant *msp4* sequence types associated with rickettsemia in individuals, and thus in the group of lambs as a whole, are consistent with the highly dynamic transmission of *A. phagocytophilum* strains around the flock. It is unlikely that the progression of detected sequence types observed in most lambs simply reflects a series of ephemeral infections, given that the ability of *A. phagocytophilum* to persist in sheep for weeks or months is well recog-

nized (11, 30, 31). Rickettsemia is not a constant feature of this persistence but rather occurs in recurrent waves interspersed with periods when at least the vast majority of rickettsiae are cleared from the blood (11). Recent work quantifying infection kinetics in experimentally infected sheep revealed that rickettsemic waves lasted between 9 and 22 days (11) with periods of up to 12 days when no organisms were detected in the blood. The repeated but sporadic detection of rickettsemia in our study, for example, as seen for *msp4* sequence type A in lamb 6019, is in keeping with this pattern. However, other experimental studies have demonstrated that the kinetics described above can be disrupted if other infecting *A. phagocytophilum* strains are present (29, 33). Given the number of *msp4* sequence types encountered in our study, it is very likely that lambs were simultaneously infected with multiple *A. phagocytophilum* strains; hence, interstrain interactions, affecting the infection kinetics of each, would be expected. The potential competitive nature of these interactions has been raised previously (33). However, exploration of this phenomenon among naturally infected animals requires the monitoring of specific *A. phagocytophilum* genotypes, which was not attempted in the current study.

Partial *msp4* sequence comparison has previously been used as a basis for the inference of phylogenetic relationships among *A. phagocytophilum* strains (8). However, when the 21 novel sequences were incorporated into phylogenetic inferences, robust support for the branching orders in the resultant reconstructions was lacking. Thus, estimation of the relative degree of evolutionary relatedness among strains, and the influence of this relatedness on the transmission dynamics observed, as previously studied for *Anaplasma marginale* (23, 24), a species closely related to *A. phagocytophilum*, was not feasible. The application of additional high-resolution *A. phagocytophilum* genotyping tools (4) may provide a solution to this problem.

The high anti-*A. phagocytophilum* antibody titers observed in most lambs early in the study period (including before their release) probably represent maternal antibodies acquired from ewes that had spent much of the previous year grazing on the same pasture. The persistence of *A. phagocytophilum* infections, and specific antibodies, in sheep held indoors over winter has been previously demonstrated (30). The presence of these antibodies may be one of the factors that underlie the low prevalence of rickettsemic lambs during the first month of their release. The influence of acaricide treatment on the transmission of *A. phagocytophilum* among the lambs is unclear; in our study, the product used by us is reported to have a persistent effect for 6 weeks (18), and we did observe that the prevalence of rickettsemia remained low for 2 or 3 weeks after the first and second treatments and declined markedly 2 to 3 weeks after the third treatment on day 56. Indeed, all but one of the genotypes detected between days 70 and 84 had been previously encountered in the same animal, perhaps indicating a recrudescent rickettsemia in the absence of new infections.

That a surprisingly large number of *A. phagocytophilum* strains concurrently circulate in the same enzootically infected population is, perhaps, another explanation for the potential that *A. phagocytophilum* appears to have for generating extensive antigenic diversity. *A. phagocytophilum* is known to possess a repertoire of over 100 different *msp2* paralogs (12), and it is thought that expression of different members of this repertoire yields immunologically distinct variants. It has been hypothesized that the ability of a particular strain to alter its antigenicity from one generation to the next, mediated by changes in *msp2* paralog expression, underlies its ability to persist in a population of reservoir hosts despite them mounting a significant immune response (37). However, for such a hypothesis it is not entirely clear why *A. phagocytophilum* needs to maintain quite so many *msp2* paralogs. Our observations lead us to speculate that the scale of this repertoire may be the consequence of a need to circumvent not only host immune responses to current or previous infections by a specific *A. phagocytophilum* strain but also those elicited by the numerous other strains circulating in the same reservoir host population.

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