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Original article

Simultaneous infection of cattle with different *Anaplasma phagocytophilum* variants

Philip Tegtmeyer[a], Martin Ganter[a], Friederike D. von Loewenich[b,*]

[a] Clinic for Swine and Small Ruminants, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, D-30173, Hannover, Germany
[b] Department of Medical Microbiology and Hygiene, University of Mainz, Obere Zahlbacherstrasse 67, D-55131, Mainz, Germany

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ABSTRACT

Anaplasma phagocytophilum is a tick-transmitted Gram-negative obligate intracellular bacterium that replicates in neutrophil granulocytes. It causes tick-borne fever in cattle and sheep. We report here the case of a 5-year-old cow from Germany with clinically overt granulocytic anaplasmosis presenting with fever, lower limb oedema and drop in milk-yield. The herd encompassed 10 animals, 8 other animals showed subclinical infection. The strains from the 9 *A. phagocytophilum* positive cows were molecularly characterized using *ankA* gene-based and multilocus sequence typing (MLST). Seven of 9 (78%) animals were infected simultaneously with different *ankA* variants belonging to *ankA* clusters I and IV. MLST analysis also revealed the presence of multiple strain types. This could be due to co-transmission or superinfection. Hosts harboring diverse *A. phagocytophilum* strains might enable the emergence of new *ankA* variants and/or MLST sequence types via bacterial recombination.

1. Introduction

Anaplasma phagocytophilum is a Gram-negative obligate intracellular bacterium that replicates in neutrophil granulocytes (Dumler et al., 2001). It causes tick-borne fever in sheep and cattle (Atif, 2015) and febrile disease called granulocytic anaplasmosis in dogs (Carrade et al., 2009), horses (Saleem et al., 2018), cats (Lappin, 2018) and humans (Ismail and McBride, 2017). Ticks of the *Ixodes persulcatus* complex are the main vectors of *A. phagocytophilum.* In much of Europe it is transmitted by *I. ricinus,* in North America by *I. scapularis* and *I. pacificus* and in Eastern Europe and East Asia by *I. persulcatus* (Stuen et al., 2013). Clinical symptoms in cattle comprise fever, inappetence, cough, lower limb oedema, drop in milk yield and abortion (Brun-Hansen et al., 1998; Woldehiwet, 2010). Leukopenia, thrombopenia and anemia are typical laboratory findings (Pusterla et al., 1997). Direct laboratory tests are recommended as diagnostic measures, usually the microscopic demonstration of bacterial inclusions in neutrophils, so-called morulae, and the detection of species-specific nucleic acids via PCR (Silaghi et al., 2017).

A. phagocytophilum has been found in asymptomatic cattle in a wide geographical range including the Far East (Ooshiro et al., 2008). However, reports on clinically overt tick-borne fever in cattle are much more infrequent. Symptomatic infections have been described in the United Kingdom (Hudson, 1950; Wilson et al., 1964), Switzerland (Hofmann-Lehmann et al., 2004; Pusterla et al., 1998), France (Chastagner et al., 2014; Lagrée et al., 2018; Matsumoto et al., 2006), Belgium (Guyot et al., 2011), Germany (Nieder et al., 2012; Silaghi et al., 2018), Turkey (Aktas and Ozubek, 2015) and Algeria (Dahmani et al., 2015).

In Europe, *A. phagocytophilum* strains infecting cattle have been molecularly characterized by different ways. A single locus approach used partial 16S rRNA, *groEL*, *msp2* and *msp4* gene sequences (Silaghi et al., 2018). Further, three multilocus techniques were applied. One compared the phylogenies derived from 16S rRNA and *ankA* gene sequences with a multilocus sequence typing (MLST) scheme based on 7 partial housekeeping gene sequences (*pheS, glyA, fumC, mdh, sucA, dnaN, atpA*) (Huhn et al., 2014). Alternatively, 9 different loci were used (*groESL, msp4, ankA, gyrA, recG, polA, typA, pleD* and the intergenic region between APH_1099 and APH_1100) (Chastagner et al., 2014). The third method analyzed 5 variable-number tandem repeat (VNTR) regions (Dugat et al., 2014).

We report here the simultaneous infection of 9 cows with different *A. phagocytophilum* strains inferred from their *ankA* gene variants and MLST.

Abbreviations: MLST, multilocus sequence typing; NJ, neighbor-joining; NT, nontypeable; ST, sequence type

* Corresponding author at: Department of Medical Microbiology and Hygiene, University of Mainz, Obere Zahlbacherstrasse 67, D-55131, Mainz, Germany.
E-mail addresses: mail@tierarztpraxis-tegtmeyer.de (P. Tegtmeyer), martin.ganter@tiho-hannover.de (M. Ganter), friederike.loewenich@unimedizin-mainz.de (F.D. von Loewenich).

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2. Material and methods

2.1. Ethics statement

The samples were obtained as part of a routine diagnostic evaluation. Written informed consent was obtained from the owner.

2.2. Microscopy

Blood smears from EDTA-anticoagulated blood were Giemsa-stained and searched for morulae at 1000-fold magnification.

2.3. Hematology

Cell counts and hemoglobin were determined by routine methods as described (Bickhardt and König, 1985).

2.4. Sequencing

DNA from EDTA-anticoagulated blood samples was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). The 16S rRNA (Massung et al., 1998; von Loewenich et al., 2003) and the *ankA* gene (Scharf et al., 2011; von Loewenich et al., 2003) were partially amplified and sequenced as described. For MLST, 7 housekeeping genes (*pheS, glyA, fumC, mdh, sucA, dnaN, atpA*) were used as reported previously (Huhn et al., 2014). The GenBank accession numbers are shown in Table 1. Isolates containing only unambiguous nucleotides (animals A198/2 and A197/5) were submitted to the *A. phagocytophilum* MLST isolates data base hosted at PubMLST (https://pubmlst.org/aphagocytophilum/).

2.5. Phylogenetic analysis

The concatenated housekeeping gene sequences described here were compared to 314 sequences published earlier (Huhn et al., 2014; Tveten, 2014). Only sequences without ambiguous nucleotides were included. The program MEGA X version 10.0.5 was used for phylogenetic analysis (Kumar et al., 2018). The concatenated allele sequences were codon-aligned by ClustalW applying the PAM (Dayhoff) matrix. Tree construction was achieved by the neighbor-joining (NJ) method using the Jukes-Cantor model with the complete deletion option. Bootstrap analysis was conducted with 1000 replicates.

3. Results

3.1. Case-presentation

In 2012, we described a congenital infection with *A. phagocytophilum* in a calf in northern Germany nearby Hannover (Henniger et al., 2013). In 2013, granulocytic anaplasmosis was diagnosed in another 5-year-old cow (A198/2) from another farm in the administrative district of Hannover that had grazed in an adjacent pasture of the dam from 2012. The herd was highly-tick infested and had shown a severe drop in milk-yield in the last weeks. The index case (A198/2) was febrile with 39.7 °C (norm 38.0–39.0 °C) and presented a severe swelling of the left foreleg. A blood smear from EDTA-anticoagulated blood was Giemsa-stained and searched for morulae at 1000-fold magnification. Inclusions of *A. phagocytophilum* were found in 50% percent of the neutrophils (Fig. 1).

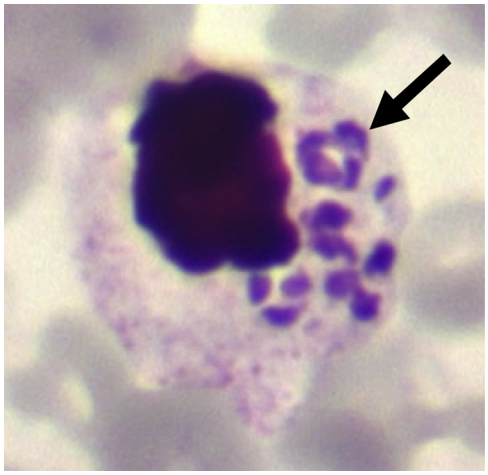

Fig. 1. Morulae (arrow) of *Anaplasma phagocytophilum* in the Giemsa-stained blood smear of the index case (A198/2). Magnification: 10 × 100.

The herd encompassed 10 animals. Except for the index case, they were found to be afebrile, but were blood-sampled as well. Morulae were found in 6 of them (60%), but in less than 1% of the neutrophils (Table 2). In the differential cell counts 5 of the 10 (50%) animals showed a leukopenia of less than 4 G/l. Neutrophil granulocytes (< 0.8 G/l) were reduced in 5 of the 10 (50%) cows and thrombocytes (< 200 G/l) in 8 (80%) of them. Erythrocyte count and hemoglobin were not affected.

The index case (A198/2) was treated with 20 mg/kg oxytetracycline for 3 days (first day i.v., second and third day i.m.) and once with 2.2 mg/kg flunixin-meglumin i.v. 0.5 mg/kg eprinomectine was topically applied once against tick-infestation in all 10 cows and the animals were housed over the following autumn and winter. After the first treatment with oxytetracycline the general condition of the index case (A198/2) markedly improved. The frequency of morulae in the blood smear decreased within 2 days to less than 1%. After treatment and housing the milk-yield of the whole herd increased.

3.2. S rRNA gene

A PCR amplifying the 16S rRNA gene was positive in 9 of the 10 animals. 497 bp of the 16S rRNA gene were analyzed. All animals were infected with the same 16S rRNA gene variant (GenBank accession number M73220).

3.3. ankA gene

Five *ankA* gene clusters I –V have been described so far (Majazki et al., 2013; Scharf et al., 2011). The partial *ankA* sequences analyzed here comprised 520 bp (cluster I) and 535 bp (cluster IV), respectively. In total, 5 different *ankA* variants were found, 3 belonging to cluster I (I_1, I_2 and I_3) and 2 to cluster IV (IV_1 and IV_2). Variant I_1 was present in 4 cows, whereas variants I_2 and I_3 infected 1 animal each. Finally, 1 of the cows harbored variants I_1 and I_2 simultaneously, which was reflected by the respective ambiguous nucleotides in the chromatograms (Table 3).

Variant IV_1 was found in 4 animals and variant IV_2 in 3 of them.

Table 1
GenBank accession numbers.

Gene	Accession numbers
16S rRNA	MK542843 – MK542851
ankA cluster I	MH987691 – MH987697
ankA cluster IV	MH997016 – MH997024
pheS	MH987457 – MH987465
glyA	MH987219 – MH987227
fumC	MH987100 – MH987108
mdh	MH987338 – MH987346
sucA	MH987576 – MH987584
dnaN	MH986981 – MH986989
atpA	MH986862 – MH986870

Table 2
Breed, age and hematological findings in the 10 cows.

Animal	Breed	Age (years)	Percent infected neutrophils	Leukocytes (G/l) norm 4.0 -10.0	Neutrophils (G/l) norm 0.8 -5.0	Thrombocytes (G/l) norm 200 - 800	Erythrocytes (T/l) norm 5.0 -10.0	Hemoglobin (g/l) norm 80 - 150
A198/2*	Fleckvieh	5	50	2.6	0.83	193	5.79	85
A197/1**	Holstein-Friesian x Fleckvieh	4	–	5.7	0.80	380	5.91	98
A197/2	Holstein-Friesian x Fleckvieh	4	–	4.1	0.37	104	7.28	122
A197/3	Red Holstein	7	< 1	3.0	0.69	123	5.32	98
A197/4	Holstein-Friesian	7	< 1	3.1	0.82	149	6.29	100
A197/5	Holstein-Friesian	7	< 1	2.1	0.59	159	4.92	88
A197/6	Fleckvieh	7	< 1	4.2	0.29	173	5.75	101
A197/7	Fleckvieh	7	< 1	2.1	0.16	106	5.02	91
A197/8	Fleckvieh	7	–	5.3	1.09	364	6.31	93
A197/9	Holstein-Friesian x Fleckvieh	7	< 1	5.0	1.40	152	6.67	117

* index case.
** 16S rRNA gene PCR negative.

Table 3
Nucleotide exchanges between the 3 cluster I *ankA* variants.

Animal	119*	167	271	277	284	290	298	376	425	Variant
A198/2	G	A	A	T	C	G	A	A	C	I_1
A197/2	G	A	A	T	C	G	A	A	C	I_1
A197/3	G	A	A	T	C	G	A	A	C	I_1
A197/7	G	A	A	T	C	G	A	A	C	I_1
A197/6	G	G	G	C	A	A	A	G	A	I_2
A197/9	G	**R**	**R**	**Y**	**M**	**R**	A	**R**	**M**	I_1, I_2
A197/5	A	G	G	C	T	G	G	A	C	I_3

* Nucleotide position in the alignment of 520 bp of cluster I *ankA* variants.

Table 4
Nucleotide exchanges between the two cluster IV *ankA* variants.

Animal	18*	56	Variant
A198/2	T	G	IV_1
A197/2	T	G	IV_1
A197/7	T	G	IV_1
A197/9	T	G	IV_1
A197/3	G	C	IV_2
A197/5	G	C	IV_2
A197/6	G	C	IV_2
A197/4	**K**	**S**	IV_1, IV_2
A197/8	**K**	**S**	IV_1, IV_2

* nucleotide position in the alignment of 535 bp of cluster IV *ankA* variants.

Table 5
Distribution of the *ankA* cluster I and cluster IV variants in the different animals.

Animal	Cluster I	Cluster IV
A198/2	I_1	IV_1
A197/2	I_1	IV_1
A197/3	I_1	IV_2
A197/4	–	IV_1, IV_2
A197/5	I_3	IV_2
A197/6	I_2	IV_2
A197/7	I_1	IV_1
A197/8	–	IV_1, IV_2
A197/9	I_1, I_2	IV_1

Two cows were infected with both variants IV_1 and IV_2 (Table 4). Seven animals contained cluster I as well as cluster IV variants. In contrast, 2 animals did not harbor any of the cluster I variants, but both cluster IV variants (Table 5). However, the exact composition of the sequences with double peaks in the chromatograms remains unknown, because we directly sequenced the PCR products instead of cloning them before.

3.4. MLST

Generally, different sequences of a given locus were ascribed a unique, but arbitrary allele number and each unique combination of alleles was assigned a sequence type (ST). A ST could not be allocated in 7 of the 9 (78%) samples, because they showed ambiguous nucleotides in up to 6 of the 7 loci (Table 6). ST 189 and ST 195 found in animals A198/2 and A197/5 have not been described before. The concatenated 7 housekeeping gene sequences were 99.8–100% identical to each other. When those without ambiguous nucleotides were compared to sequences published earlier (Huhn et al., 2014; Tveten, 2014), they clustered together with samples from cattle and European bison (Fig. 2).

For the *pheS* locus, 3 of 9 cows showed the same ambiguous nucleotides at positions 75 and 183. A similar observation was made for the *glyA* locus as 5 of 9 animals had the nucleotide R at positions 270 and 358. The ambiguous nucleotide M at position 99 in the *fumC* locus

Table 6
ST and allele numbers of the seven housekeeping genes used for MLST.

Animal	ST	*pheS*	*glyA*	*fumC*	*mdh*	*sucA*	*dnaN*	*atpA*
A198/2	189	101	2	66	51	11	7	3
A197/2	NT*	101	2	66	NT	11	7	NT
A197/3	NT	NT	NT	NT	8	NT	NT	NT
A197/4	NT	NT	NT	NT	8	NT	NT	NT
A197/5	195	21	2	68	8	11	76	19
A197/6	NT	7	2	7	8	11	NT	NT
A197/7	NT	7	NT	3	8	63	NT	NT
A197/8	NT	NT	NT	NT	8	NT	NT	NT
A197/9	NT	7	NT	3	8	NT	NT	NT

* NT = nontypeable.

found in 3 of the 9 cows could be resolved by the simultaneous presence of *fumC* alleles 3 and 68. The same was true for the *mdh* locus (1 animal harboring both alleles 8 and 51) and for the *sucA* locus (4 animals harboring both alleles 11 and 63). However, the 6 ambiguous positions in the *dnaN* locus in 6 of the 9 cows could not be explained by the simultaneous presence of the *dnaN* alleles 7 and 76 infecting the rest of the animals. A similar observation was made for the *atpA* locus showing ambiguous nucleotides in 7 of the 9 cows.

4. Discussion

One of the *A. phagocytophilum* infected cows reported here (A198/2) was clinically ill. In the blood smear, inclusions of *A. phagocytophilum* were found in 50% of the neutrophils. Eight other animals were asymptomatically infected. Six of them (75%) showed morulae in the blood smear. However, in these animals bacterial inclusions were found in less than 1% of the neutrophils. In a study analyzing a dairy cattle herd over one pasture season, the occurrence of fever was associated with the presence of morulae probably reflecting a high bacterial load. However, morulae were occasionally observed in afebrile cows as well (Silaghi et al., 2018). Therefore, in general, there seems to be no strict correlation between the microscopic detection of *A. phagocytophilum* and clinical symptoms. The concatenated 7 housekeeping gene sequences reported here were 99.8–100% identical to each other. Thus, homologous *A. phagocytophilum* strains circulated in the herd and a correlation between clinical symptoms and the respective genetic variants was not found.

All animals analyzed here were infected with the same 16S rRNA gene variant (GenBank accession number M73220) which was initially reported from sheep in Scotland (Anderson et al., 1991) and is often found in sheep (Huhn et al., 2014; Stuen et al., 2002) and cattle (Huhn et al., 2014; Nieder et al., 2012; Silaghi et al., 2018). Single locus 16S rRNA gene-based typing of *A. phagocytophilum* has been proven to not reliably define *A. phagocytophilum* genotypes (Bown et al., 2009; Bown et al., 2007; Casey et al., 2004; Huhn et al., 2014; Scharf et al., 2011; von Loewenich et al., 2003). This is further underlined here, because the cows were found to harbor several *ankA* and MLST types despite the presence of only one 16S rRNA gene variant.

The simultaneous infection of a calf from the same geographic region as the cows studied here with two *ankA* variants belonging to clusters I and IV has been described before (Henniger et al., 2013). Interestingly, 7 of the 9 (78%) cows in our study harbored both, cluster I and cluster IV variants, raising the possibility that co-transmission or superinfection with different variants is more the rule than the exception. In roe deer, red deer and ticks the infection with *ankA* variants from different clusters has been described as well (Huhn et al., 2014; Jouglin et al., 2017; Scharf et al., 2011). In a study comprehensively investigating the co-infection rate in roe deer from France 34/56 (61%) animals harbored 2 or even 3 *ankA* variants belonging to clusters II, III and IV (Jouglin et al., 2017). This probably reflects the high tick-exposure of domestic and wild ruminants. Whether multiple *ankA* variants are co-transmitted or whether the animals are superinfected remains to be demonstrated. Needle inoculated sheep held in a tick-free environment developed recurrent bacteremia with *A. phagocytophilum*

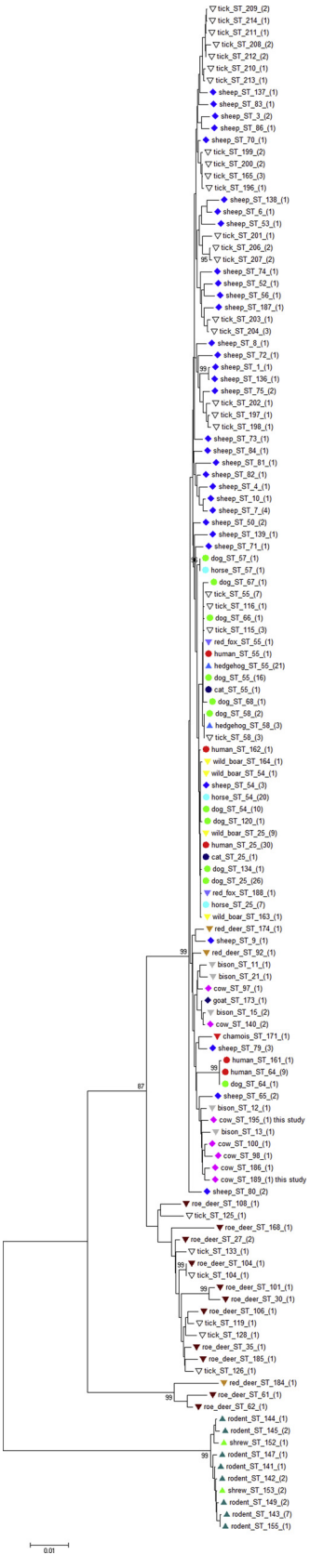

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Fig. 2. NJ phylogenetic tree calculated from the concatenated housekeeping gene sequences.
Tree construction was achieved by the NJ method using the Jukes-Cantor matrix with the complete deletion option. Bootstrap values lower than 87% are not shown. The scale bar indicates the number of nucleotide substitutions per site. The final data set contained 2877 positions. Identical ST are displayed only once per species. The number in parenthesis indicates the frequency with which the respective ST was found.
Symbols: ● human, ● dog, ● horse, ● cat, ◆ sheep, ◆ cattle, ◆ goat, ▼ roe deer, ▼ red deer, ▽ European bison, ▼ wild boar, ▼ chamois, ▼ red fox, ▲ hedgehog, ▲ vole, ▲ shrew, ▽ tick.

for up to 1 year, but did not show clinical symptoms (Thomas et al., 2012). For cattle, this has not been proven yet in an experimental setting, but naturally infected cows have been found to harbor the same genetic variant over several months (Lagrée et al., 2018). Thus, persistently infected cattle might be superinfected by other *A. phagocytophilum* variants and could serve as reservoir hosts for bovine tick-borne fever.

According to the MLST analysis the animals were infected with several *A. phagocytophilum* strains, 7 of them simultaneously. This has been observed before at other loci (*typA, msp4, pleD, regG, polA*) in 13/31 (42%) of French cattle showing double peaks in the respective chromatograms (Lagrée et al., 2018). In our study, only in 2 cows a ST could be allocated to the infecting strain. However, these animals harbored both, cluster I and cluster IV *ankA* gene variants. Further, the 2 animals that contained only variants belonging to cluster IV, showed 2 different cluster IV variants, IV_1 and IV_2. This means that all 9 cows analyzed here were simultaneously infected with a set of different *A. phagocytophilum* strains. Previously, it has been shown that 14 of 34 (41%) analyzed *I. ricinus* ticks were infected with more than one ST (Huhn et al., 2014). Thus, the possibility exists that multiple *A. phagocytophilum* variants are co-transmitted. Hosts harboring diverse *A. phagocytophilum* strains might enable the emergence of new *ankA* variants and/or MLST sequence types via bacterial recombination. It has been shown before that the formation of the different *ankA* clusters probably have been arisen by recombining various DNA fragments (Majazki et al., 2013).

5. Conclusion

We show here the simultaneous circulation of multiple genetic variants of *A. phagocytophilum* in a German cattle herd. Via bacterial recombination new variants might evolve that escape the host's immune system. This would allow the persistence of the infection at the herd level and has implications for control measures.

Declarations of interest

None.

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