

## Tick-Borne Transmission of Two Genetically Distinct *Anaplasma marginale* Strains following Superinfection of the Mammalian Reservoir Host<sup>∇</sup>

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**Strain superinfection affects the dynamics of epidemiological spread of pathogens through a host population. Superinfection has recently been shown to occur for two genetically distinct strains of the tick-borne pathogen *Anaplasma marginale* that encode distinctly different surface protein variants. Superinfected animals could serve as a reservoir for onward transmission of both strains if the tick vector is capable of acquiring and transmitting both strains. Whether competition among strains during development within the tick vector, which requires sequential invasion and replication events, limits colonization and subsequent transmission to a single strain is unknown. We tested this possibility by acquisition feeding *Dermacentor andersoni* ticks on a reservoir host superinfected with the genetically distinct St. Maries and EMΦ strains. Although the St. Maries strain consistently maintained higher bacteremia levels in the mammalian host and the EMΦ strain had an early advantage in colonization of the tick salivary glands, individual ticks were coinfecting, and there was successful transmission of both strains. These results indicate that a genetically distinct *A. marginale* strain capable of superinfecting the mammalian host can subsequently be cotransmitted and become established within the host population despite the presence of an existing established strain.**

Superinfection occurs when a second, genetically distinct pathogen strain infects a host already carrying a primary strain of the same pathogen species. Superinfection allows the second strain to become established in the host and compete with the primary strain for onward transmission. For antigenically variant microbial pathogens this requires that the second strain encode a unique set of variants. This hypothesis has recently been tested and accepted using the tick-borne bacterial pathogen *Anaplasma marginale* as a model (5). Strains of *A. marginale* that encode distinct variants of the immunodominant surface protein Msp2 are capable of superinfection despite the establishment of long-term infection and broad immunity against the variants encoded by the primary strain. Consequently, superinfected animals maintain an infection with two genetically distinct strains and, in theory, serve as reservoir hosts of both strains for subsequent tick transmission to naïve animals.

Whether two distinct strains can be simultaneously acquired by the tick vector and subsequently transmitted is unknown, and this represents a gap in our knowledge that is relevant to pathogen strain structure in reservoir host populations. If only a single pathogen strain can be acquired and transmitted, then the new strain (either generated by genetic change or introduced) needs to have a significant competitive advantage to become established in the reservoir host population. Conversely, transmission of both strains to naïve animals would

progressively expand the representation of the second strain in the population.

*A. marginale* undergoes complex development within the tick, and pathogen strain fitness for transmission is affected at at least two steps. The initial infection and a first round of intracellular replication occur within the tick midgut epithelium (7, 9, 10). The ability to enter and replicate in the midgut epithelium has been shown to be a determinant of *A. marginale* strain transmission fitness in the tick *Dermacentor andersoni* (16). Whether simultaneous acquisition of two *A. marginale* strains results in competition between the strains has not been tested, but entry of a first *A. marginale* strain into the midgut has been postulated to prevent establishment of infection by a second strain via a process termed “infection exclusion” (3). The second event required for transmission is pathogen entry into and replication within the tick salivary glands (7, 9–11). There are strain-specific differences in colonization within the salivary gland, and the strain transmission fitness of *D. andersoni* is also affected at the level of the salivary gland (16). Consequently, competition between strains at either step (initial midgut invasion and replication or subsequent invasion and replication within the salivary gland) may limit transmission to a single *A. marginale* strain despite tick acquisition feeding on a reservoir host superinfected with two strains. Here we describe testing this hypothesis and present the findings in the context of determinants of pathogen strain transmission fitness and the implications for strain structure within the reservoir host population.

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### MATERIALS AND METHODS

**Strain superinfection.** The St. Maries (2, 4) and EMΦ (12) strains are genetically distinct strains with nonoverlapping genomic repertoires of *msp2* pseudo-gene alleles (13), and they have been shown to establish superinfection regard-

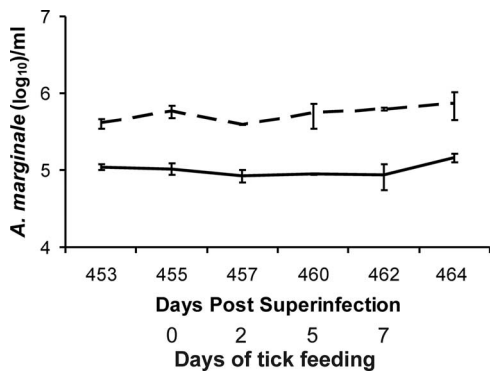


FIG. 1. Strain-specific bacteremia levels during acquisition feeding. The numbers of organisms of the EMΦ strain (solid line) and the St. Maries strain (dashed line) per milliliter of blood were determined by quantitative PCR during the period of tick acquisition feeding on animal 995 (15 months following the initial superinfection).

less of which strain is the primary infecting strain and which strain is the superinfecting strain (5). Superinfection was established by primary infection of naïve Holstein calves with the St. Maries strain, followed >12 months later by tick transmission of the EMΦ strain. The presence and level of each strain during superinfection in the reservoir host and at the time of tick acquisition feeding were determined by strain-specific PCR and strain-specific quantitative PCR as described in detail below.

**Strain-specific detection and quantification.** DNA was extracted either from washed erythrocytes using a Puregene DNA isolation kit (Gentra) and the whole-blood protocol or from individual tick salivary gland pairs as previously described (11, 14). Strain-specific PCR targeted the *msp1α* gene, which has a set of repeats that vary in sequence and number near the 5' end (1). The St. Maries strain-specific primers were forward primer 5'-CAGCAGAGTATGTCTCCTC C-3' and reverse primer 5'-CATTGGAGCGCATCTCTTGC-3', and the EMΦ strain-specific primers were forward primer 5'-TAGCAGAGTGTGTGTCG-3' and reverse primer 5'-GCCTGACCGCTTTGAGATGA-3'. These strain-specific primer sets amplify fragments that are different sizes, as follows: for strain St. Maries, 319 bp; and for strain EMΦ, 179 bp. Infection status was determined using PCR followed by Southern hybridization with the *msp1α* gene as a probe. Digoxigenin-labeled probes were synthesized using a Roche PCR DIG probe synthesis kit and the *msp1α* primers described above. The PCR consisted of one cycle of 96°C for 5 min, followed by 30 cycles of 96°C for 30 s, 62°C for 30 s, and 72°C for 1 min and then a final extension at 72°C for 7 min. The PCR fragments were separated on a 1.2% agarose gel prior to Southern blotting as previously described (13). The level of infection with each strain was quantified by real-time PCR using the strain-specific *msp1α* primers described above and Taqman FAM-490 strain-specific probes. The St. Maries probe was 5'-TCAGCTGATAGCTC GTTAGCGGGT-3', and the EMΦ probe was 5'-CCAGCTGATAGCTCGTC AGCGAGT-3'. Each strain was assayed independently of the other strain, and then the results were compared after adjustment using each standard curve. A standard curve was constructed for each strain-specific assay by cloning the *msp1α* gene from each strain into the PCR-4 TOPO vector (Invitrogen Corporation, Carlsbad, CA). The assay consisted of one cycle of 95°C for 10 min, followed by 50 cycles of 95°C for 30 s, 60°C for 15 s, and 72°C for 1 min and a final extension at 72°C for 7 min.

**Tick acquisition feeding and transmission.** Adult male *D. andersoni* ticks (*n* = 360) from the Reynolds Creek colony were allowed to attach and feed on animal 995 for 48 h (*n* = 60), 96 h (*n* = 60), and 168 h (*n* = 240). The presence of both strain St. Maries and strain EMΦ and the levels of these strains in the blood during the tick acquisition feeding period were determined using the PCR methodology described above. Following tick detachment, ticks were held at 26°C and 94% relative humidity for at least 120 h to ensure that there was complete digestion of the ingested bloodmeal and then placed on naïve (*msp5* PCR-negative and competitive-inhibition enzyme-linked immunosorbent assay-seronegative) calves for transmission feeding. Ticks that acquisition fed for 48 or 96 h were transmission fed under separate patches on the same Holstein calf (animal 5854) for 48 h and then removed. Ticks that acquisition fed for 168 h were transmission fed on one of four Holstein calves (animals 5877, 5882, 5890, and 5896; 60 ticks per calf) for 168 h. Salivary gland pairs were dissected from individual ticks immediately following transmission feeding, and DNA was iso-

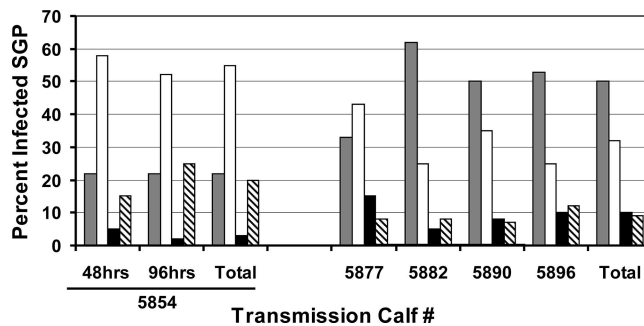


FIG. 2. Percentages of ticks infected with single *A. marginale* strains or coinfecting following acquisition feeding on a superinfected reservoir host. The percentages of *D. andersoni* tick salivary gland pairs (SGP) in each infection category are indicated as follows: gray bars, coinfecting ticks; open bars, EMΦ-infected ticks; black bars, St. Maries-infected ticks; cross-hatched bars, uninfected ticks. For calf 5854 two groups of ticks (60 ticks each) were used; one group was acquisition fed for 48 h, and the other group was acquisition fed 96 h. For calves 5877, 5882, 5890, and 5896 ticks (60 ticks/animal) were acquisition fed for 168 h.

lated for determination of the presence and levels of each *A. marginale* strain as described above. The calves were monitored for infection using microscopic examination of Giemsa-stained blood smears, *msp5* PCR amplification, and seroconversion using the competitive-inhibition enzyme-linked immunosorbent assay (15). The presence and level of each strain following transmission were determined by PCR-Southern analysis and quantitative real-time PCR as described above.

**RESULTS**

**Bacteremia levels of *A. marginale* strains in the superinfected reservoir host during tick acquisition feeding.** As previously reported, calves superinfected with the St. Maries and EMΦ strains contain higher levels of the St. Maries strain early after superinfection regardless of whether the St. Maries strain was the primary strain (*n* = 4; the level of St. Maries was 3 times higher) or the superinfecting strain (*n* = 4; the level of St. Maries was 5.2 times higher) (5). Consistent with this difference, animal 995 used for tick acquisition feeding had higher bacteremia levels with the St. Maries strain than with the EMΦ strain following superinfection (data not shown). The higher St. Maries strain bacteremia was confirmed at the time of acquisition tick feeding, 15 months following initial superinfection, and the levels were approximately fivefold greater than the EMΦ levels (Fig. 1).

**Prevalence of *A. marginale* strain-specific and strain coinfection in ticks.** PCR amplification followed by Southern blotting provides highly sensitive and specific detection of *A. marginale* (6) and therefore was used to determine the infection status of the fed adult ticks (data not shown), which were assigned to one of four groups: uninfected, infected with the St. Maries strain, infected with the EMΦ strain, or infected with both strains (Fig. 2). Overall, 80% of the ticks acquisition fed for 48 and 96 h were infected, while 91% of the ticks fed for 168 h were infected. Of the infected ticks, 40% were coinfecting with both the St. Maries and EMΦ strains, while 47% carried a single strain. The percentage of ticks coinfecting with both strains was significantly greater for ticks acquisition fed for 168 h than for ticks fed for a shorter time (*P* < 0.0001, Pearson chi-square test). Comparison of ticks infected with a single

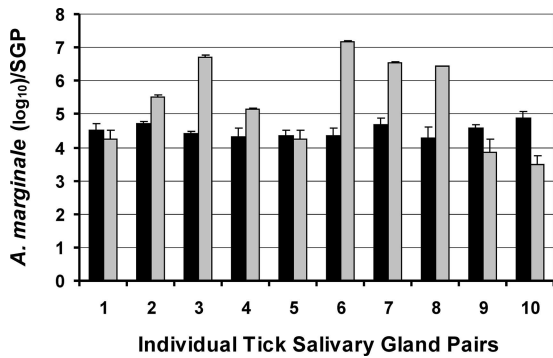


FIG. 3. Strain levels within coinfecting ticks. Salivary gland pairs (SGP) from individual coinfecting ticks that had acquisition and transmission fed for 168 h were analyzed by quantitative PCR in triplicate. The black bars indicate EMΦ strain levels, and the gray bars indicate St. Maries strain levels.

strain (Fig. 2) revealed that the EMΦ strain was predominant regardless of the duration of acquisition feeding ( $P < 0.0001$ ).

***A. marginale* levels in coinfecting ticks.** Pathogen levels within tick salivary glands were determined using quantitative real-time PCR. The coinfecting ticks initially acquisition fed for 48 and 96 h had higher levels of the EMΦ strain ( $8.9 \times 10^3$  bacteria per salivary gland pair) than of the St. Maries strain, whose levels were below the lower limit ( $10^3$  bacteria per salivary gland pair) of the linear range for the real-time PCR assay. For ticks that had acquisition fed for 168 h, there was no significant difference between the levels of the strains, and the levels of both strains consistently were greater than  $10^3$  bacteria per salivary gland pair (Fig. 3). The levels during coinfection were not significantly different from the levels previously reported for infections with a single strain (6, 16). Ticks acqui-

sition fed on calves singly infected with the St. Maries strain using the same tick colony and a 168-h feeding period contained  $10^3$  to  $10^7$  organisms per salivary gland pair, a range similar to the range observed in the current experiment (Fig. 3).

**Transmission of *A. marginale* to naïve animals.** All calves ( $n = 4$ ) on which ticks transmission fed for 168 h developed an infection with both *A. marginale* strains by 2 weeks after the transmission feeding. Tracking the bacteremia of each strain following transmission again revealed significantly higher ( $P < 0.0005$ , Wilcoxon matched-pair signed-rank test) levels of the St. Maries strain than of the EMΦ strain (Fig. 4). Ticks initially acquisition fed for 48 or 96 h were transmission fed for 48 h, and consistent with the requirement for a longer feeding time, the animal did not develop infection during the 75-day post-feeding observation period and did not seroconvert.

## DISCUSSION

The hypothesis that tick acquisition feeding on a reservoir host carrying two genetically distinct *A. marginale* strains results in competition between the strains, leading to exclusion of one strain to the advantage of the second strain, is rejected. This suggests that following the initial penetration of a new strain into a reservoir host population in an area of *A. marginale* endemicity by superinfection, both the new strain and the existing strain can be cotransmitted to naïve animals, thus expanding the representation of the new strain within the population. This is consistent with epidemiologic observations indicating that animals carrying two genetically distinct strains can be detected (12, 13). There is, however, a notable caveat to this conclusion: both strains used in the present study were highly transmissible strains that efficiently infect and replicate in adult male *D. andersoni* ticks and thus the analysis may not

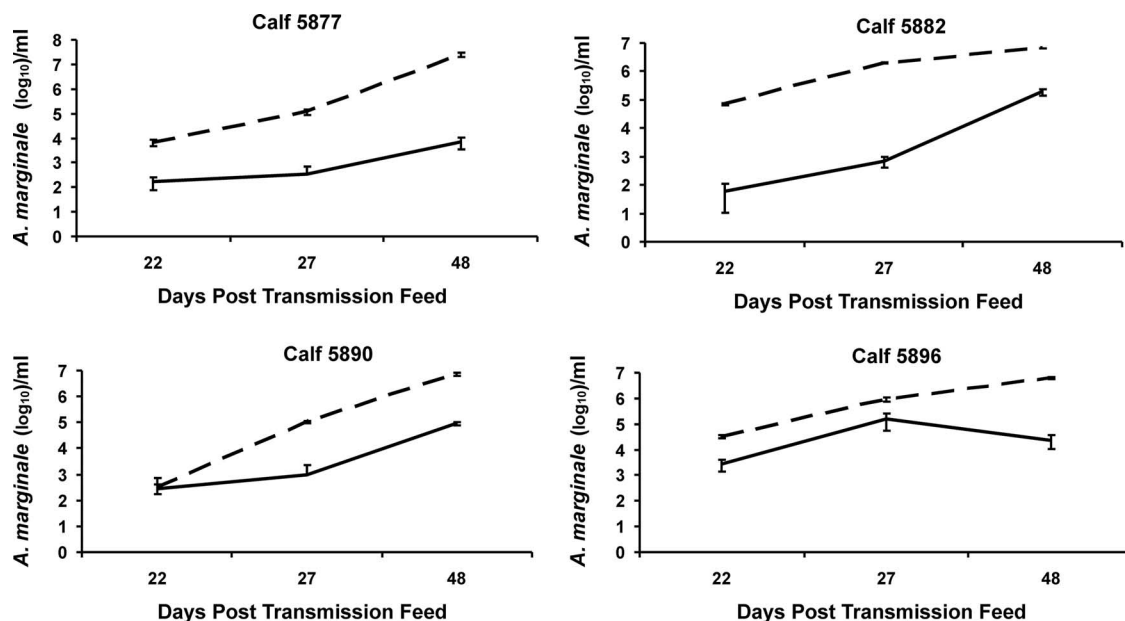


FIG. 4. Strain levels within coinfecting calves following tick transmission. Strain-specific bacteremia levels were determined during acute infection using quantitative PCR in triplicate. The data for the EMΦ strain are indicated by the solid lines, and the data for the St. Maries strain are indicated by the dashed lines.

represent competition between strains with markedly different levels of transmission fitness. The evidence for strain-specific differences in transmission fitness includes evidence from field studies in which a specific strain was the predominant strain in the reservoir host population despite the presence of multiple circulating strains and evidence from experimental studies in which replication within the tick and subsequent transmission differ significantly for different strains (12, 16). However, none of the previous transmission fitness studies examined the competition between strains when two strains were acquired simultaneously.

Although both the St. Maries and EMΦ strains are highly transmission-fit strains, quantitative analysis of infection in both the mammalian reservoir host and the tick vector revealed significant fitness differences. The St. Maries strain consistently maintains a higher level of bacteremia during persistent infection than the EMΦ strain. The difference was originally noted at early time points following strain superinfection, regardless of which strain was the primary strain and which strain was the superinfecting strain, and it was observed in the present study both in the persistently infected animal (15 months after superinfection) used for tick acquisition feeding and in the naïve animals following transmission. This consistent observation for a total of 13 calves, which was based on data for both strain superinfections and simultaneous strain transmission resulting in coinfection, indicates that the St. Maries strain has a competitive advantage within the reservoir host (5). Nonetheless, the higher level of bacteremia did not result in either a significantly higher percentage of ticks singly infected with the St. Maries strain than with the EMΦ strain or significantly higher numbers of the St. Maries strain within ticks. In contrast, there is evidence that the EMΦ strain has an early advantage within the tick, as indicated by the higher percentage of ticks singly infected with the EMΦ strain than with the St. Maries strain following the shorter acquisition feeding times (48 and 96 h). However, there was no difference following the longer 168-h acquisition feeding period, and both strains were efficiently transmitted. Whether more pronounced strain-specific differences in the bacteremia level in the reservoir host or the infection prevalence and level in the tick vector result in strain-specific differences in transmission remains unanswered.

Transmission of *A. marginale* requires initial invasion and a first round of replication in the tick midgut epithelium, followed by a second round of invasion, replication, and development of infectivity in the salivary gland (7, 9–11). The mechanisms underlying these events are poorly understood, as is the minimum time needed for replication to an infectious dose and development of a fully infectious phenotype in the salivary gland. The timing required for this development is critical, as shown by the lack of transmission when ticks were acquisition fed for up to 96 h and then transmission fed for 48 h. In contrast, longer periods of tick feeding are associated with consistent transmission, and there are data correlating increased efficiency of transmission with increased duration of tick transmission feeding (8, 9). The temporal requirement for tick feeding is epidemiologically important as adult male ticks are the primary vector, initially feeding on an infected reservoir host and then, following spermatogenesis, transferring among additional individual hosts in search of female ticks, resulting in interrupted feeding and transmission (8–10, 17; D. Stiller, M. E. Coan, W. Goff, L. W. Johnson, and T. C. McGuire,

presented at the Eighth National Veterinary Hemoparasite Disease Conference, St. Louis, MO, 10 to 12 April 1989). Consequently, both tick behavior and the reservoir host population structure can affect transmission efficiency.

In summary, there is clear evidence for simultaneous acquisition and transmission of genetically distinct *A. marginale* strains following superinfection in the reservoir host. This supports the importance of superinfection for establishment of a new strain within an infected reservoir host population in an area of *A. marginale* endemicity. Whether the introduced strain is maintained at a lower or higher prevalence within the population than the original strain depends on the relative transmission fitness of each strain. The present study did not resolve whether simultaneous transmission of two strains was due to coinfecting ticks that transmitted both strains or to a population of ticks individually infected with either the St. Maries or EMΦ strain; however, both of these transmission scenarios have the same epidemiological impact. While both scenarios seem plausible, resolution of this question awaits further transmission studies using only coinfecting ticks. Similarly, this study did not directly address the “infection exclusion” hypothesis that ticks that acquire a first strain by feeding on an infected reservoir are prevented from acquiring a second strain and subsequently transmitting the two strains (3). Determining the mechanism and relative efficiency of these events is essential for obtaining a better understanding of how strains emerge and compete under natural vector-borne transmission conditions.

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