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Strain Diversity in Major Surface Protein 2 Expression during Tick Transmission of *Anaplasma marginale*

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Specific major surface protein 2 (MSP2) variants are expressed by *Anaplasma marginale* within the tick salivary gland and, following transmission, are expressed during acute rickettsemia. In previous work, we have shown that a restricted pattern of MSP2 variants is expressed in the salivary glands of *Dermacentor andersoni* ticks infected with the South Idaho strain of *A. marginale*. Now we demonstrate that the identical restriction does not apply to two other strains of *A. marginale*, and that different variants are also expressed when the same strain is transmitted by different *Dermacentor* spp. This indicates that antigenic diversity among strains is maintained in tick transmission and may be a significant constraint to MSP2 vaccine development.

Anaplasma marginale is a tick-borne pathogen of cattle that causes severe anemia during acute rickettsemia (13). Individuals that survive acute disease remain persistently infected and serve as reservoirs for transmission (4, 25). Persistent infection is characterized by sequential cycles of rickettsemia, each composed of a progressive, logarithmic increase in rickettsemia followed by a precipitous decrease (3, 4, 9). In each cycle *A. marginale* that express novel structural and antigenic variants of the immunodominant outer membrane protein major surface protein 2 (MSP2) emerge (7, 8). These variants, typified by amino acid substitutions, deletions, and insertions in the central hydrophilic region of MSP2, express unique B-cell epitopes that are recognized, not at the time of emergence, but only following control of each rickettsemic cycle (7). Thus, the antigenic structure of the *A. marginale* populations continually changes throughout persistent infection and ixodid ticks feeding during persistence ingest a heterogeneous population of variants that differ over time and among individual animals within a herd (15).

Following ingestion in the bloodmeal by feeding ticks, *A. marginale* undergoes a complex developmental cycle of replication within midgut epithelium and gut muscle cells, culminating in the development of infective stages in the tick salivary gland (10, 11, 22). In studies using *Dermacentor andersoni* acquisition and transmission of the South Idaho strain of *A. marginale*, we discovered that a restricted set of MSP2 variants were expressed within the salivary gland and transmitted to naïve cattle (19). The same MSP2 salivary gland variants (SGV) were expressed within ticks that had acquired *A. marginale* infection by feeding on different individual calves at different time points, feeding during both acute and persistent rickettsemia, and feeding on rickettsemic blood containing distinctly different MSP2 variants (19). The restriction of MSP2 variant heterogeneity in the salivary gland is significant, as *A. marginale* expressing these variants were transmitted to cattle and subsequently composed the acute rickettsemia (19). This suggested that, in contrast to the antigenic heterogeneity in persistently infected cattle, the restricted set of transmitted MSP2 SGV could provide a stable target for vaccine development.

A. marginale strains isolated from acute disease outbreaks can be distinguished genetically and differ in the antigenic structure of the major surface proteins, virulence, and tick transmissibility (1, 5, 14, 21, 24). However, all examined strains contained the polymorphic *msp2* multigene family and expressed structurally variant MSP2 during each of the rickettsemic cycles in persistent infection (2, 6, 7, 15–19). Vaccine development based on a restricted set of MSP2 SGV would require that only these variants, or at least a limited number of variants, be expressed by the salivary gland stages of multiple, and ideally all, *A. marginale* strains. Do different strains of *A. marginale* express identical MSP2 SGV within the tick? We addressed this question by comparing the sequences of *msp2* transcripts expressed in the salivary glands of *D. andersoni* ticks fed on cattle infected with the St. Maries (Idaho) strain with the MSP2 SGV1 and SGV2 expressed by the South Idaho strain of *A. marginale*. Both strains are naturally transmitted by *D. andersoni* ticks and have been shown to be experimentally transmitted by the *D. andersoni* laboratory stock isolated in Idaho and used in this experiment (4, 5). Calf 787 was infected by intravenous inoculation of a stabilate containing 10^{10} erythrocytes infected with the St. Maries strain. Giemsa-stained blood smears were examined daily to monitor the development of acute rickettsemia, and when rickettsemia levels reached 10^9 infected erythrocytes per ml, 250 laboratory-reared adult male *D. andersoni* ticks were placed in an orthopedic stockinette and allowed to attach and acquisition-feed for 7 days. The ticks were removed and incubated for an additional 7 days at 26°C with 90 to 98% relative humidity and a 14-h photo period. To stimulate development of the infective stage in the salivary gland (10, 11, 22), the ticks were allowed to attach and feed on an uninfected calf, 789, for 3 days. Ticks were then removed and total RNA was extracted from isolated salivary glands, as previously described (19). Transmission to calf 789 was confirmed by microscopic detection of *A. marginale*-infected erythrocytes, and total RNA was extracted from whole blood collected on the first day of microscopically detectable rickettsemia, using Trizol (Bio-Rad Laboratories), as described previously (8). Total RNA was reverse transcribed with random

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FIG. 1. Amino acid sequence alignment of the hypervariable regions from MSP2 SGV1 and SGV2 from the St. Maries (StM) strain of *A. marginale*. Areas of amino acid substitutions, insertions, and deletions are indicated by a white background, areas of amino acid identity have a black background, and grey shading indicates conservative amino acid substitutions.

hexamers, and *msp2* cDNA was amplified by using PCR (2, 8). The full-length transcript was amplified by using forward and reverse primers from the conserved 5′ and 3′ ends (7, 19). To amplify only the *msp2* hypervariable region, primers derived from the conserved regions that flank the central, hypervariable 595-bp region of *msp2* were used (7, 8). The primer sequences, amplification conditions, cloning into pCR2.1, and sequencing were all as previously reported (7, 8, 19). *D. andersoni* adult male ticks of the same stock were acquisition-fed on an uninfected calf and were handled identically and served as negative controls. No *msp2*-specific amplicons were identified with salivary gland RNA from these control ticks.

Variant *msp2-sgv* full-length transcripts were identified in the St. Maries strain-infected salivary glands by sequencing 37 independently derived cDNA clones. Consistent with previous results from studies with the Florida and South Idaho strains, MSP2 polymorphism in transcripts of the St. Maries strain was localized to the central hypervariable region (amino acids 185 to 280, based on the predicted amino acid sequence of pCKR11.2 *msp2* [16]). The MSP2 SGV hypervariable region sequences encoded by the two predominant transcripts, defined as composing more than 10% of the cDNA clones, were designated St. Maries MSP2 SGV1 and SGV2 (Fig. 1). Neither these nor the minor variants (fewer than 10% of the total

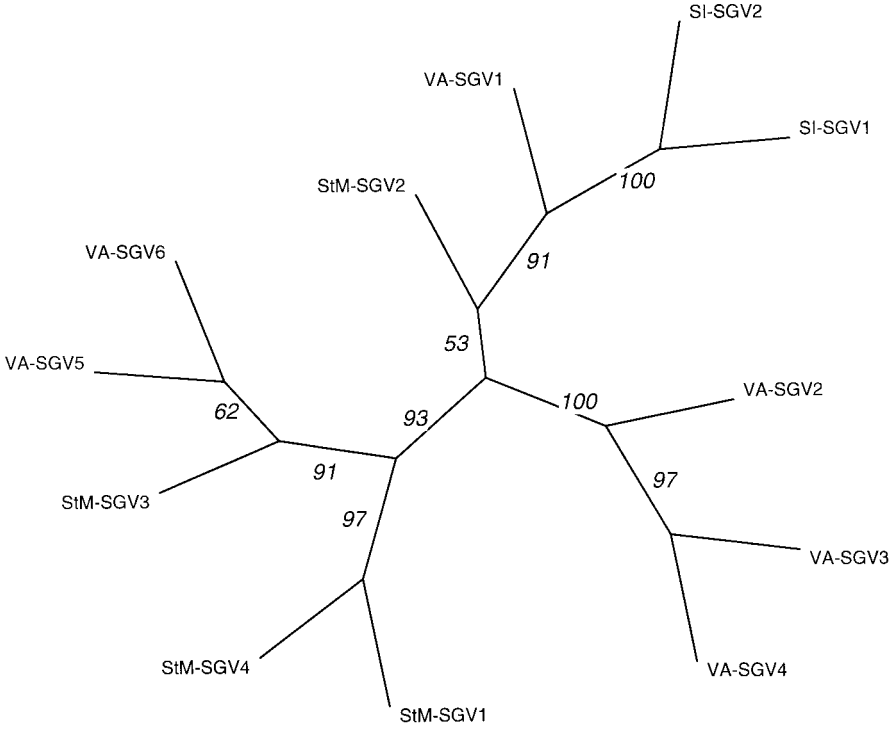

FIG. 2. A phylogram of MSP2 SGV types expressed by the South Idaho (SI), St. Maries (StM), and Virginia (VA) strains in *D. andersoni*, based on predicted amino acid sequences. The SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE programs in the PHYLIP phylogenetic inference package were used for the derivation of the data used in the phylogram (6). Bootstrap values from 100 analyses are shown at the branch points of the tree.

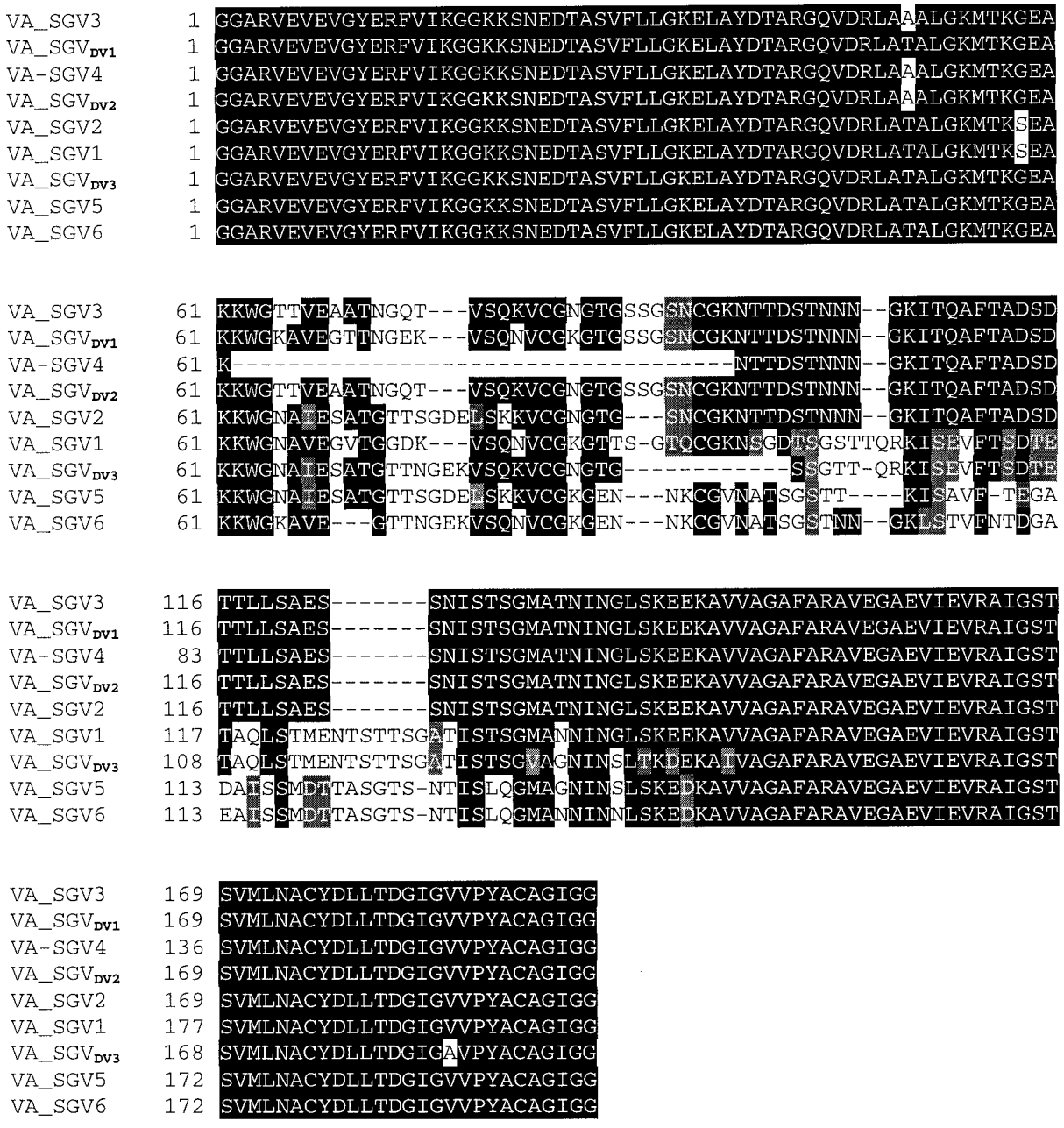

FIG. 3. Amino acid sequence alignment of the MSP2 SGV hypervariable regions expressed by the Virginia (VA) strain in *D. andersoni* (SGV1 to SGV6) and *D. variabilis* (SGV$_{Dv}$1 to SGV$_{Dv}$3). Areas of amino acid substitutions, insertions, and deletions are indicated by a white background, areas of amino acid identity have a black background, and grey shading indicates conservative amino acid substitutions.

clones sequenced) encoded proteins identical to the previously reported South Idaho strain MSP2 SGV1 and SGV2 (19). The most similar are the St. Maries MSP2 SGV2 and the South Idaho MSP2 SGV1, which share 90% identity in the approximately 200 amino acids composing the central hypervariable region. Thus, two strains, both isolated from acute outbreaks in Idaho and naturally transmitted by *D. andersoni*, expressed distinctly different MSP2 SGV in the same stock of *D. andersoni*. In addition, the St. Maries strain expressed multiple heterogeneous variants, unlike the restricted expression of only two closely related variants by the South Idaho strain (19).

Analysis of the *A. marginale* transcripts expressed during acute rickettsemia of calf 789, following tick transmission of the St. Maries strain, revealed that 10 of 11 clones had the St. Maries MSP2 SGV1 sequence. Expression of identical MSP2 in both the salivary gland and in the bloodstream also occurs in the South Idaho strain (19). This pattern is notably different from that shown by tick-transmitted *Borrelia hermsii*, in which there is a switch in the expressed surface coat between organisms in the salivary gland and those in the blood of the mammalian host following transmission (20). For *A. marginale*, expression of new variants of MSP2 is not seen until later in acute rickettsemia, presumably reflecting immune selection of MSP2 variants (15, 19).

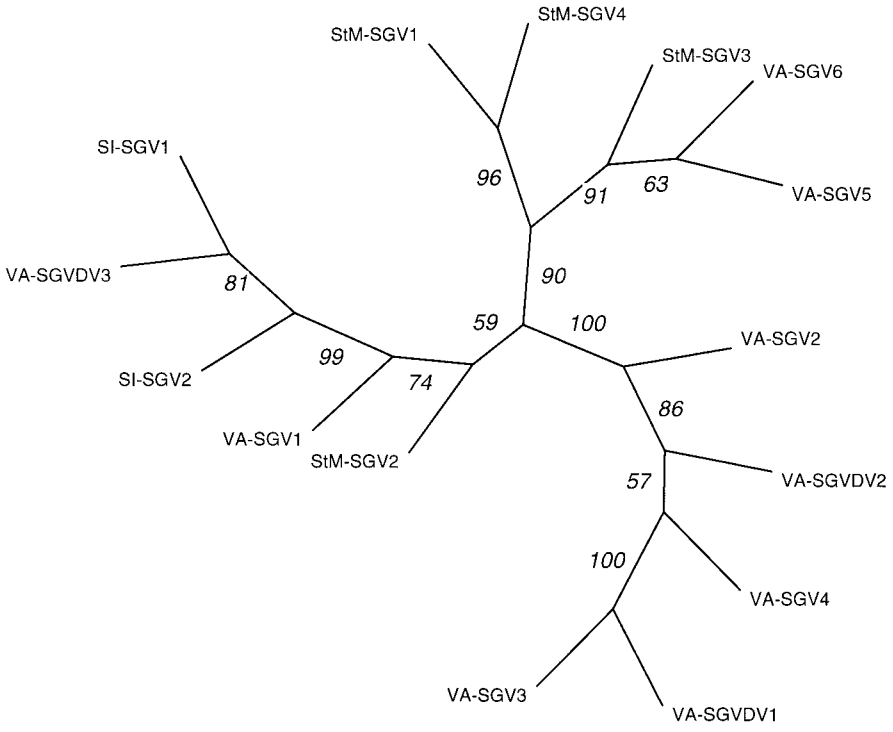

FIG. 4. A phylogram of MSP2 SGV types expressed by the *A. marginale* South Idaho (SI), St. Maries (StM), and Virginia (VA) strains in *D. andersoni* (SGV1 to SGV4) and in *D. variabilis* (SGVDV1 to SGVDV3). The distance value determination, tree construction, and calculation of the bootstrap values were done as described for Fig. 2.

The differences in MSP2 SGV between the South Idaho and St. Maries strains raised the question of whether specific hypervariable region sequences in each strain were associated with development within the tick salivary gland. If so, the MSP2 SGV hypervariable regions would be expected to cluster by strain. To increase the number of strain-specific sequences for analysis, the acquisition feeding and development of infective *A. marginale* stages in the salivary gland was repeated with a third *D. andersoni*-transmissible strain. Calf 794 was infected by intravenous inoculation of 10^{10} erythrocytes infected with the Virginia strain and was monitored, as described above, for development of rickettsemia. Adult male *D. andersoni* ticks were acquisition-fed on calf 794, and following incubation and transmission feeding to stimulate development of infectivity, RNA was isolated from the salivary glands. The *msp2*-specific cDNA clones were obtained and sequenced, as described previously for the South Idaho strain (19). Again, none of the Virginia strain MSP2 SGV was identical to any of those expressed by either the South Idaho or the St. Maries strain. Comparison of the two South Idaho MSP2 SGV, four St. Maries MSP2 SGV (major and minor variants), and the six Virginia MSP2 SGV by using a phylogram based on the hypervariable region amino acid sequences revealed that the expressed MSP2 SGV do not segregate by strain (Fig. 2). This is illustrated by comparison of Virginia MSP2 SGV1, which is more similar to the South Idaho MSP2 SGV1 and SGV2 and to St. Maries MSP2 SGV2 than to any other Virginia MSP2 SGV (Fig. 2). Furthermore, examination of the MSP2 SGV encoded by each strain using BestFit analysis to detect small regions of conservation and the Genetics Computer Group shuffle program to test significance of conserved oligopeptides (program manual for the Genetics Computer Group package, Genetics Computer Group, Madison, Wis.) failed to identify any strain-specific oligopeptide motifs within the hypervariable region (data not shown).

Overall, the polymorphism encoded within the SGV-MSP2 hypervariable regions of the three tick-transmitted strains examined is similar to that expressed by a single strain during rickettsemic cycles in persistently infected cattle (7, 8, 19). The MSP2 central hypervariable region has been shown to encode surface-exposed B-cell epitopes (2, 7, 8), and even very closely related MSP2 proteins, sharing more than 90% identity in amino acid sequence, are antigenically distinct (2). Furthermore, these MSP2 epitopes are highly immunogenic and development of variant-specific antibody correlates with clearance of the expressed variant (7, 17, 23). Although all expressed MSP2 variants, including the MSP2 SGV, have highly conserved N- and C-terminal regions (2, 7, 19), these regions are hydrophobic membrane domains with minimal surface exposure (7). Consequently, the central region diversity in expressed MSP2 SGV indicates that a vaccination strategy targeted solely against the MSP2 SGV is unlikely to protect against the numerous strains transmitted by ticks within endemic regions.

To test whether the vector tick species affects the specific MSP2 SGV expressed by an *A. marginale* strain, the sequences of the *msp2-sgv* transcripts expressed by the Virginia strain within the salivary glands of adult male *Dermacentor variabilis* were compared to those expressed by this strain within the salivary glands of adult male *D. andersoni*. Like *D. andersoni*, *D. variabilis* is a natural vector of *A. marginale*, and the Oklahoma State University laboratory stock used in this experiment has been shown to transmit the Virginia strain (12, 22). Using separate stockinettes, 250 adult males of each *D. andersoni* and *D. variabilis* were acquisition-fed for the same 7-day period on calf 794, infected with the Virginia strain as described above.

In vitro incubation, stimulation of infectivity by transmission feeding, isolation of total RNA from isolated salivary glands, generation of *msp2* cDNA clones, and sequencing were done as described previously (8, 19). Three expressed MSP2 SGV were identified in the Virginia strain within *D. variabilis* and were designated MSP2 SGV_{Dv} 1, 2, and 3. None of these was identical to any of the six Virginia strain MSP2 SGV expressed within *D. andersoni* (Fig. 3). This observation is consistent with the selective or inductive role of the tick vector and suggests that the influence of the tick may differ between vector species. However, no tick species-specific motifs were identified by comparison of the Virginia strain MSP2 SGV expressed in *D. andersoni* and *D. variabilis*. Furthermore, comparison of the Virginia strain MSP2 SGV sequences in *D. variabilis* with all the MSP2 SGV sequences from the three strains (St. Maries, South Idaho, and Virginia) in *D. andersoni* indicated that expressed MSP2 SGV sequences did not cluster by tick species (Fig. 4).

In contrast to the findings in our original study, using the South Idaho strain, which has restricted expression of only two very closely related MSP2 SGV (19), in this study both the St. Maries and Virginia strains expressed multiple, heterogeneous MSP2 SGV. This heterogeneity and lack of tight restriction was observed in the Virginia strain in both vector species examined. The basis for this difference among strains is currently unknown but may reflect strain-specific selection for certain MSP2 SGV sequences or differences in regulation of gene expression. Using the sequences and methodology reported here, we were unable to identify specific hypervariable region sequences common to multiple MSP2 SGV that could associate with a required function in the salivary gland and we could not detect clustering of the expressed MSP2 SGV by either organism strain or vector species. The regulation of *msp2* gene expression has not been completely defined. Recently, *msp2* genes have been shown to be expressed as part of a four-gene operon (A. F. Barbet, A. Lundgren, J. Yi, F. R. Rurangirwa, and G. H. Palmer, submitted for publication). Whether *A. marginale msp2* can also be expressed individually under the direct control of a *msp2*-specific promoter is unknown; however, this has been reported for the *msp2* orthologue (p44) in the closely related agent of human granulocytic ehrlichiosis (26). This raises the possibility that expression of specific *A. marginale msp2* genes may be regulated either individually or as part of an operon. Determining whether differential regulation of gene expression occurs within the tick vector and if it varies between strains is important for understanding the basis of MSP2 expression within the tick salivary gland.

Nucleotide sequence accession numbers. The *msp2* nucleotide sequences have been assigned the GenBank accession numbers AF107766 to AF107767 and AF227261 to AF227271.

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