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Distinctly different *msp*2 pseudogene repertoires in *Anaplasma marginale* strains that are capable of superinfection

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Abstract

Lifelong persistent infection of cattle is a hallmark of the tick transmitted pathogen *Anaplasma marginale*. Antigenic variation of Major Surface Protein 2 (MSP2) plays an important role in evasion of the host immune response to allow persistence. Antigenic variation of MSP2 is achieved by gene conversion of pseudogenes into the single operon linked expression site and the diversity of variants is defined by the pseudogene repertoire. Once an animal is persistently infected with one strain of *A. marginale*, infection with a second strain (superinfection) is rare. However, we recently detected animals superinfected with different strains of *A. marginale* and hypothesized that the *msp*2 pseudogene repertoire would be distinct in these superinfecting strains, consistent with encoding different sets of surface variants. Five strains of *A. marginale* were selected in order to identify and compare *msp*2 pseudogene content; these included strains with similar and different *msp*1α genotypes, and genotypes that were representative of the strains that were found in the superinfected animals. Southern blot analysis of strains associated with superinfection revealed distinctly different *msp*2 banding patterns, in contrast to a pattern suggesting identical pseudogene content among related strains not associated with superinfection. Indeed, targeted sequence analysis of *msp*2 pseudogenes showed identical pseudogene repertoires in genotypically closely related strains and varying amounts of dissimilarity in the pseudogene repertoire in strains with distinctly different *msp*1α genotypes, but totally different *msp*2 pseudogene repertoires between the strains that were found in superinfected animals. This finding supports the hypothesis that the occurrence of superinfection reflects the differences in the *msp*2 repertoire and corresponding diversity of variants.

Keywords: Major surface protein; *msp*1α; Outer membrane protein

1. Introduction

Anaplasmosis, the most prevalent tick-borne disease of cattle and other ruminants worldwide is caused by the intraerythrocytic rickettsia, *Anaplasma marginale* (Losos, 1986). Animals that survive an acute infection are unable to clear the organism and remain persistently infected for life. This persistence is marked by recurring subclinical peaks of rickettsemia (Eriks et al., 1993). The *A. marginale* population in each peak expresses a new variant(s) of Major Surface Protein 2 (MSP2), an immunodominant, antigenically variant molecule (Palmer et al., 1988; French et al., 1999). This event is thought to be instrumental for evasion of the host immune response (Palmer et al., 2000; French et al., 1998). *Msp*2 is part of a multigene family that is composed of a single operon-associated expression site (ES) and several functional pseudogenes (Brayton et al., 2005; Palmer et al., 1994; Barbet et al., 2000). The functional pseudogenes are truncated versions of *msp*2 containing the central hypervariable region (HVR) and shortened portions of the conserved 5′ and 3′ ends

Abbreviations: *msp*2, major surface protein 2; *opag*, operon-associated gene; PCR, polymerase chain reaction; VRR, variable repeat region; HVR, hypervariable region; ES, expression site; gDNA, genomic DNA.

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(Brayton et al., 2001). The pseudogenes may be recombined into the ES in whole or in part to generate combinatorial diversity in *msp*2 (Brayton et al., 2001, 2002). As a result, the specific variant population generated during infection is defined by the number and sequence of the *msp*2 pseudogene repertoire.

Previous studies in herds naturally infected with *A. marginale* have shown that individual animals usually carry only one strain despite the presence and transmission of genotypically distinct strains within the population (Palmer et al., 2001, 2004). This is consistent with the prevention of superinfection with a second strain following development of a broad repertoire of MSP2 variant-specific immune responses against the first *A. marginale* strain. As a consequence, we would predict that only strains with markedly different *msp*2 pseudogene repertoires would be capable of superinfection due to the relative lack of cross-reactive immune responses. In epidemiologic studies, *A. marginale* strains are genotyped using the single copy *msp*1α (Allred et al., 1990), a stable genetic marker that does not undergo variation during infection of the mammalian host or within the ixodid tick vector (Palmer et al., 2001; Allred et al., 1990). Diversity among the *msp*1α genes from different strains is determined based on the number and sequence of tandem repeats, each 84–87 bp long, positioned near the 5′ end (Allred et al., 1990). Recently we observed high genotypic diversity in an *A. marginale* endemic herd in Kansas in which the genotypes were grouped into three families: B, DE, and EM, based on the similarity within each family in the type of *msp*1α repeats (Palmer et al., 2004). In addition, we identified the presence of different *A. marginale* strains, referred to as superinfection, in each of five individual animals within the herd. We hypothesized that these superinfecting *A. marginale* strains would have distinctly different *msp*2 pseudogene repertoires. To test our hypothesis, we examined the *msp*2 pseudogene complement in five strains from this Kansas herd in order to assess the relatedness of the *msp*2 pseudogene repertoire and demonstrate that *A. marginale* strains associated with superinfection have markedly different pseudogene repertoires.

2. Experimental procedures

2.1. A. marginale organisms

Based on the *msp*1α genotype from a herd at Kansas State University a group of five *A. marginale* infected animals was selected (Palmer et al., 2004). Two strains with identical genotype (5B), one strain with a closely related genotype (6B), and two strains with completely different genotypes (EM∅ and 6DE) were selected. Animal numbers and their respective genotypes are 3201:EM∅, 6192:5B, 7072:5B, 9038:6B, 8416:6DE. Because two strains carry the identical genotype, animal numbers will be used to distinguish between strains. To confirm the genotype five splenectomized calves were sub-inoculated with fresh infected blood from the Kansas herd animals. The complete genome sequence has been determined for the Saint Maries strain, and this strain was used as a reference for this study, GenBank accession no. CP000030 (Brayton et al., 2005).

2.2. Confirmation of msp1α genotype of Kansas strains

Blood was drawn at the acute rickettsemic peak between 6% and 21% PPE, washed six times with PBS and kept frozen until genomic DNA (gDNA) was prepared using the Puregene® genomic DNA purification kit (Gentra Systems). The *A. marginale* genotype was determined by sequencing of the variable repeat region (VRR) in the 5′ region of the gene. Amplification of the *msp*1α tandem repeats was done with primers flanking the VRR and PCR Master (Roche) as previously described (Palmer et al., 2001). The forward primer was 1AF1: 5′-tcaacactcgcaaccttgg and the reverse primer was PH1AR: 5′-tgcttatggcagacatttcc. The number of repeats and their respective sequences were confirmed by cloning into pCR4-TA-TOPO and sequencing using the Big Dye kit and an ABI 3100 capillary sequencer. Sequences were compiled and analyzed using the Vector NTI Suite software package (InforMax, North Bethesda, MD). The genotype was assigned by a letter from A to Z or α to Φ for each unique repeat following the convention reported by different authors (Allred et al., 1990; Palmer et al., 2001; De la Fuente et al., 2003; Futse et al., 2003, and by García-García et al., 2004). In order to exclude errors, with addition or loss of repeats, during PCR amplification, *Fsp*I digested gDNA was used for Southern blot hybridizations with a specific probe spanning the repeat region of *msp1*α (Fig. 1). The probe was generated by PCR using the primers VRRf: 5′-catttccatatactgtgcag and VRRr: 5′-cttggagcgcatctctctgcc.

2.3. Southern analysis of A. marginale genomic DNA

Genomic DNA from the five Kansas strains was digested with *Fsp*I, separated on a 0.8% agarose gel and subsequently transferred to a nylon membrane. The blots were prehybridized in Dig Easy Hyb buffer (Roche) at 42 °C for at least 2 h in hybridization bags. Digoxigenin-labeled probes were produced by using the PCR DIG Probe Synthesis kit (Roche). Hybridization buffer containing the digoxigenin-labeled probe

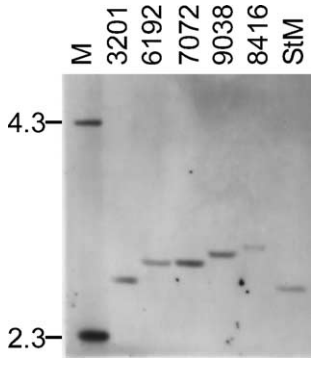

Fig. 1. Southern blot analysis using a VRR probe on *Fsp*I digested gDNA to genotype strains from the Kansas herd. *Fsp*I cut DNA fragment sizes are: 3201 (EMΦ), 2700 bp; 6192 and 7072 (5B), 2870 bp; 9038 (6B), 2960 bp; and 8416 (6DE), 3045 bp.