TRANSMISSION DYNAMICS OF GENETICALLY DISTINCT STRAINS OF *Anaplasma marginale* FOLLOWING SUPERINFECTION

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of MARIA FERNANDA B. M. GALLETTI find it satisfactory and recommended that it be accepted.

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Once, I read "*A vida não é feita de momentos em que voce respirou e sim daqueles que tiraram-te o fôlego*" …"*Life is not made of moments that allow you to breath but moments that take your breath away!*" and it is exactly how I felt through the two years I lived in Pullman. This experience was absolutely fascinating.

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Abstract

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Strain superinfection occurs when a second pathogen strain infects a host already carrying a primary strain. *Anaplasma marginale* superinfection occurs when the second strain encodes a unique variant surface repertoire as compared to the primary strain and its epidemiologic consequences depend on the relative transmission efficiency of the two strains. Following strain superinfection in the reservoir host, we tested whether the presence of two strains that differed in transmission efficiency altered the transmission phenotypes as compared to single strain infections. *Dermacentor andersoni* males were fed on calves superinfected with the *A. marginale ss centrale* vaccine strain (low transmission efficiency) and the *A. marginale* St. Maries strain (high transmission efficiency). As expected, ticks most commonly acquired both strains (co-infection) although single infections with each strain were also detected. There was no significant difference in the percentage of ticks that acquired the St. Maries strain as compared to the *A. centrale* vaccine strain although most ticks developed higher levels of infection with the St. Maries strain. The St. Maries strain was transmitted to naïve calves by ticks fed either on superinfected calves or on calves solely infected with the St. Maries strain. In

contrast, the *A. centrale* vaccine strain was not transmitted by either singly or co-infected ticks. These results suggest that the observed strain predominance in endemic regions can be mediated by the transmission efficiency of specific strains regardless of occurrence of superinfection.

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DEDICATION

Thru all the years of my life, everything I accomplish is and will always be dedicated to my family, my mother, my father and my sister that support, assist and encourage me, everyday, to do the best all step of the way.

With all my love.

INTRODUCTION

Strain superinfection occurs when a second pathogen strain infects a host already persistently infected with a primary strain. Superinfection has been reported for pathogens ranging from small genome RNA viruses, such as human immunodeficiency virus and hepatitis C virus, to complex parasites such as *Trypanosoma brucei* (1, 7, 11) The epidemiologic consequences of superinfection depend on the subsequent competition between the two strains for onward transmission to a new host. We address this question using by studying transmission of *Anaplasma marginale*, a tick-borne bacterial pathogen which establishes persistent infection in the mammalian reservoir hosts (domestic and wild ruminants) (4) and for which the basis for strain superinfection has been recently reported (5).

A. marginale strain superinfection occurs when the second strain encodes a different repertoire of the antigenically variable outer membrane protein, designated Major Surface Protein-2 (MSP2) (2), that allows the second strain to escape immunity generated against the primary strain. Once superinfection is established in the reservoir host, both strains are maintained and can be acquired and transmitted by the tick vector (5). If both strains were equally acquired and transmitted by the tick, this would be predicted to generate a reservoir host population carrying multiple strains with roughly equal representation of the diverse strains. However epidemiologic studies have indicated that a single pathogen strain predominates within spatially and temporally defined reservoir host populations. This may be explained by, at least, two alternative hypotheses. The first hypothesis is that ticks feeding on a superinfected animal are less efficient in either simultaneous acquisition of the two strains or the subsequent replication of the two strains,

so ticks fed on a superinfected animal are at a competitive disadvantage as compared to ticks fed on a host infected with a single strain. Mechanistically this may result from division of the overall capacity of the tick to support infection and replication so that both strains are at reduced levels in terms of either the percentage of fed ticks that acquire infection or the number of organisms per infected tick. The alternative second hypothesis is that there is no interaction between the strains within the vector and that there is simply preferential transmission of the most fit individual strain.

To address these hypotheses, we selected two pathogen strains with different intrinsic transmission efficiency. The St. Maries strain of *A. marginale* is highly transmissible by adult males of *Dermacentor andersoni* (9, 10). This efficiency is manifested in a high infection rate (the % of fed ticks that acquire the pathogen), replication to high titer in the salivary gland, and consistent transmission to naïve animals. In contrast, the Israel vaccine strain is significantly less efficiently acquired and transmitted using the same *D. andersoni* colony. We predicted that these two strains would superinfected, given the presence of at least one distinctly different *msp2* allele between the two strains. Superinfection of the reservoir host with these two strains would then permit testing the interaction between two strains within the vector following tick acquisition feeding and determining if this interaction affected development and subsequent transmission of the more highly transmissible St. Maries strain. Herewith we report testing these hypotheses and discuss the results in the context of the epidemiological consequences of pathogen strain superinfection.

MATERIALS AND METHODS

Pathogen strains, tick vectors, and animal hosts: The transmission efficiency of both the St. Maries strain and the *A. marginale* ss. *centrale* vaccine strain by *Dermacentor andersoni* has been previously reported. The specific-pathogen free Reynolds Creek colony of *D. andersoni* was used in all transmission experiments. Age-matched male Holstein calves were used in all experiments; all calves were negative for *A. marginale* by MSP5 C-ELISA prior to initiation of the experiments.

***A. marginale* superinfection of vaccine strain infected animals:** Four calves (nos. 6171, 6175, 6187, 6188) were infected by intravenous inoculation with 10^8 organisms of the vaccine strain. As a control, calf (no. 6170) was inoculated at the same time but using 10^8 organisms of the St. Maries strain of *A. marginale*. All calves became infected as determined by microscopic examination of Giemsa-strained blood smears and by MSP5 C-ELISA seroconversion. Following progression to persistent infection (bacteremia $\leq 10^7$ organisms/ml), three of the vaccine strain infected calves (nos. 6175, 6187, 6188) were exposed to ticks infected with the St. Maries strain. Briefly, adult male *D. andersoni* infected with the St. Maries strain were allowed to transmission feed (n=50 ticks/calf) for 7 days. Superinfection (the presence of both strains) was detected by strain-specific PCR of blood and quantified using strain-specific quantitative PCR. Both assays are described in detail in sections below. The remaining vaccine strain infected calf (no. 6171) and the St. Maries strain infected calf (no. 6170) were maintained as controls for transmission of single strains.

Transmission following tick feeding on superinfected animals: Uninfected *D. andersoni* adult males were allowed to attach and acquisition feed on one of the three groups of animals: (i) those superinfected with the vaccine strain and the St. Maries strain (nos. 6175, 6187, 6188); (ii) infected with only the vaccine strain (no. 6171); and (iii) infected with only the St. Maries strain (no. 6170). Ticks were acquisition fed for 7 days and engorged ticks removed and incubated for an additional 7 days at 26°C at 93% relative humidity with a 12-h photoperiod. Cohorts of the ticks from each animal were then dissected and DNA extracted from individual salivary glands and midguts to determine the presence and quantity of each strain (6). The remaining ticks were used for transmission feeding on naïve, C-ELISA seronegative calves (32004, 32023, 32001, 31991 32008) to determine if the presence of the vaccine strain interfered with the transmission of the wild-type St. Maries strain. Following 7 days of transmission feeding, ticks were removed, salivary glands obtained by dissection, and DNA isolated as described below. Transmission to the naïve animals was monitored by microscopic examination of Giemsa-stained blood smears, strain-specific PCR amplification of DNA isolated from blood, and C-ELISA seroconversion.

Strain-specific PCR: Dissected tick salivary glands were collected in cell lysis buffer and digested with proteinase K overnight at 37°C followed by incubation at 65°C for 2 h. DNA was isolated from blood samples and tick tissues using the Puregene DNA isolation kit (Gentra Systems). For strain-specific PCR, the locus containing the *msp1α* gene was targeted; the St. Maries strain has a prototypical *msp1α* gene while the *A. marginale* ss. *centrale* vaccine strain is divergent in this locus. The St. Maries strain was amplified using

forward primer 5' tgcttatggcagacatttccat 3' and reverse primer was 5' gggaaagaggacaaccacaca 3', generating a 155bp amplicon. The vaccine strain was amplified using forward primer 5' tgcagttgagaagttccgtatca 3' and reverse primer 5' tgttgccttagctgggtcaat 3', generating a 122bp amplicon. The identities of the strain-specific amplicons were confirmed by sequencing in both directions. To enhance sensitivity of detection of infected ticks, strain-specific amplicons were probed by Southern analysis using a digoxigenin-labeled probe. The probes were generated using the strain-specific primers listed above and the PCR DIG Probe Synthesis Kit (Roche). Following hybridization overnight at 42^{o}C, the membrane was washed 3x for 15 min in 2×SSC/0.1% SDS. The first two washes were performed at room temperature and the third one at 65 °C. A final 15 min wash was done in 0.2×SSC/0.1% SDS at 65 °C. Chemiluminescent detection of the probes was achieved by using the DIG Wash and Block Buffer Kit (Roche) as described by the manufacturer and CDP-Star (Roche).

For quantification, the same strain-specific primer sites in combination with specific TaqMan probes: 5' cgtatgttacaatcaggccgccgg 3' for the St. Maries strain and 5' acatgccattattgacccagctaaggcaa 3' for the vaccine strain. The TaqMan Real Time PCR protocol was adapted from (3) with changes on the PCR target and $MgCl_2$ concentration to increase specificity. Briefly, the initial cycle was 95°C for 10 min followed by 55 cycles of 95°C for 20 s, 58°C for 10 s, and 72°C for 10 s, a final extension at 72°C for 30 s, and holding at 10°C. The real-time PCR reaction mix contained 10 mM Tris (pH 8.3), 50 mM KCl, 4.0 mM $MgCl_2$, 200 μM of each dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, 0.2 μM fluorogenic probe, and 1.25 U of AmpliTaq Gold (PE Applied Biosystems, Foster City, CA) with all reactions performed on the iCycler iQ real-time PCR detection

system (Bio-Rad, Hercules, CA). The same amplicons using the primers described above were cloned into pCR-4 TOPO vector (Invitrogen Corporation) to build the standard curve with dilutions between 10^7 and 10^2 copies of the plasmids specific for each strain. An infected *D. andersoni* salivary gland containing 10^5 *A. marginale* St. Maries strain or vaccine strain organisms were used as internal controls for repeatability among assays. Triplicates were analyzed from each sample. The number of organisms was determined by using the standard curve and presented as mean $\log_{10}$ (± standard deviation) number of organisms per salivary gland pair or per ml of blood.

RESULTS

A. marginale superinfection of vaccine strain infected animals

Calves persistently infected with the *A. marginale* ss. *centrale* vaccine strain became superinfected upon transmission feeding of ticks infected with the St. Maries strain of *A. marginale*. Both strains were detected in the three challenged calves (6175, 6187 and 6188) by 31 days following the St. Maries strain challenge (Fig. 1). The calves maintained as single infection controls, 6170 and 6171, contained only the single strain, the St. Maries and vaccine strain respectively (Fig. 1). Quantitative tracking using strain-specific real-time PCR revealed no significant differences in the levels of each strain in superinfected animals as compared to the single strain infected controls over the same time period post-challenge (Fig. 2 and 3).

Tick infection following acquisition feeding on superinfected animals

Specific pathogen-free adult male *D. andersoni* were acquisition fed on the superinfected calves and the single strain control calves. During the 7 day acquisition feeding, the levels of each strain were quantified in each animal using strain-specific PCR. The mean levels during acquisition feeding were approximately 10^6 per ml of blood in all animals with no significant difference between the levels in the superinfections as compared to the single strain infections (Table 1). Within individual superinfected animals there were higher mean levels of the vaccine strain as compared to the St. Maries strain however these differences were not statistically significant (Table 1). Following acquisition feeding, the ticks were incubated at 26^{o}C for an additional 7 days to ensure complete digestion of the bloodmeal. Separate cohorts of ticks fed on each individual calf were then dissected and the presence and level of each pathogen strain in the tick salivary gland were determined. Ticks fed on the animals infected with a single strain, 6170 and 6171, were infected with only the expected strain, St. Maries and the vaccine strain, respectively (Table 2). The higher infection rate (% of fed ticks that acquire infection) for the St. Maries strain as compared to the vaccine strain is consistent with previous studies examining acquisition of these two strains from single infections (Table 2). Ticks acquisition fed on superinfected calves included all four possible classes of infection status: infected with the initial vaccine strain, infected with the superinfecting St. Maries strain, co-infected with both strains, and uninfected (Figure 4). Analyzed as a group, ticks acquisition fed on the three superinfected animals (n=353 ticks), 45% were co-infected with the two strains. These co-infected ticks were then used to test whether one strain had a competitive advantage within individual ticks when both strains were acquired. Using quantitative competition index (CI) analysis

where CI= number of St. Maries strain organisms – vaccine strain organisms/ total number of the two strains combined, the St. Maries strain significantly predominated ($p < 0.001$; Wilcoxon Rank-sum Test). This competitive advantage occurred despite the presence of lower levels of the St. Maries strain as compared to the vaccine strain in the superinfected animals during acquisition feeding (Table 2).

Transmission to naïve animals following acquisition feeding on superinfected animals

The remaining cohorts of ticks acquisition fed on individual superinfected calves were then transmission fed on individual naïve calves (n=70 ticks/animal). Ticks initially acquisition fed on calves infected with a single strain were handled identically with transmission feeding on naïve calves (n=70 ticks/animal). The St. Maries strain was transmitted using ticks acquisition fed on each of the three superinfected calves and using ticks acquisition fed on the calf infected with only the St. Maries strain (Fig. 5). The interval from tick transmission feeding until first detection of the St. Maries strain was similar among animals, all animals seroconverted within 20-30 days, and all animals developed a peak acute bacteremia of $>10^8$ organisms per ml. In contrast, there was no transmission of the *A. marginale* ss. *centrale* vaccine strain using ticks acquisition fed on either the superinfected animals or the single strain infected animal (Fig. 5). All animals remained PCR-negative for the vaccine strain throughout the 80 day observation period, representing greater than three standard deviations beyond the mean interval for detection of St. Maries strain transmission. The identity of the strain-specific amplicons detected in the calves used for acquisition feeding, in the ticks fed on either superinfected or single strain

infected calves, and in the calves following transmission were confirmed by sequencing (data not shown).

DISCUSSION

The establishment of strain superinfection following tick transmission of the St. Maries strain to animals already persistently infected with the vaccine strain is consistent with the model proposed by Rodriguez et al. (8) and experimentally shown by Futse et al. (2) in which *A. marginale* superinfection occurs when two strains encode unique *msp2* allelic repertoires. Although the complete *msp2* allelic repertoire for the vaccine strain has not yet been reported, there are clearly marked differences in this repertoire as indicated by sequencing the expression site *msp2* in the vaccine strain and comparison to the full *msp2* repertoire available in the complete genome sequence of the St. Maries strain. This is in contrast to the lack of superinfection observed among closely related strains with shared *msp2* allelic repertoires.

Superinfection of natural reservoir hosts by strains with significantly different transmission phenotypes permitted testing of the interaction among strains during acquisition by the tick and subsequent replication. Our results support rejecting the hypothesis that exposure to two strains during tick acquisition feeding adversely affects development within the vector. In contrast the results indicated that the efficient transmission phenotype of the St. Maries strain was unaffected by the presence of a second less efficiently transmitted strain and are consistent with attributing the observed predominance of a specific strain in naturally infected reservoir hosts to intrinsic

transmission efficiency. There are two important caveats to these conclusions. The first is that the vaccine strain may not faithfully represent transmission phenotypes of currently circulating wild-type *A. marginale* strains. While there are no observable differences in levels or duration of infection within the reservoir host between the vaccine and wild-type strains, whether its low efficiency of transmission (<100 fold less efficient than the St. Maries strain) is representative of wild-type strains is uncertain. However, multiple *A. marginale* strains have been isolated that have dramatically reduced transmission efficiency by *D. andersoni* and thus there is compelling evidence that there is a broad spectrum of transmission phenotypes among currently circulating strains. The second caveat is that transmission of the St. Maries strain by ticks initially acquisition fed on superinfected animals used a population of ticks that included both co-infected ticks and ticks infected with only the St. Maries strain. Thus the possibility that only the singly infected ticks were responsible for transmission cannot be excluded. However this seems unlikely given that the numbers of the St. Maries strain in the salivary gland were the same regardless of whether the tick was singly or co-infected.

The ability of ticks to acquire more than a single *A. marginale* strain has been controversial. The concept of "infection exclusion" within the tick has been evoked to explain why a single strain predominates in the reservoir host population during natural transmission. Two distinct mechanisms could underlie this concept in the tick. The first is that there is a limited capacity in terms of either permissive cell for pathogen invasion or to support subsequent replication. As the pathogen load in superinfected animals is additive, this increased load could exceed the threshold capacity and permit tick infection and replication by one strain to the exclusion of the second. Our results argue that this does not

occur and that two strains with differing transmission phenotypes may establish infection simultaneously and essentially independently. This is also supported by a study in which ticks simultaneously acquired and transmitted two *A. marginale* strains with highly transmissible phenotypes (5). A second exclusion mechanism is the induction of the tick innate defense responses following invasion of the first strain which would then prevent colonization by the second strain. How this would manifest itself temporally when ticks simultaneously acquire two strains while feeding on a superinfected animal is unclear. However this mechanism could come into play if ticks acquired the first strain feeding on one infected animal host and then, following interhost transfer, subsequently fed on a second animal infected with a distinct strain. This is compatible with the intermittent feeding and mate-seeking behavior of adult male *D. andersoni*, however its relevance to strain predominance under conditions of natural transmission remain unexplored.

Strain superinfection introduces a new strain into the reservoir host population. The consequences of this for disease epidemiology depend not only on the onward transmission fitness of the newly introduced strain but on critical determinants responsible for virulence, immunogenicity, and host range, among others. Improved understanding of shifts in pathogen strain structure within reservoir hosts will likely prove essential to better prediction and early control of new disease phenotypes.

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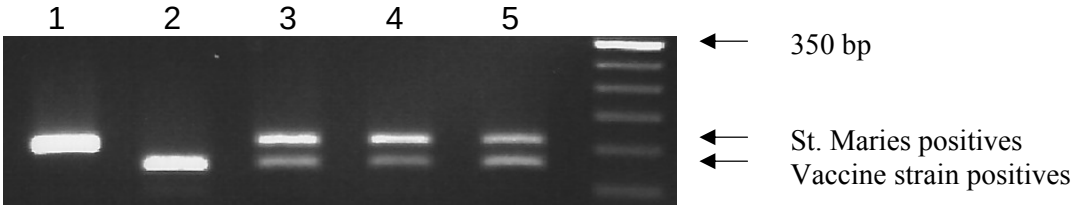

Figure 1 – Duplex PCR *msp1α* detection of the control and superinfected cows. Lane 1 – St. Maries control cow (c6170); Lane 2 – Vaccine strain control (c6171); Lanes 3, 4 and 5 – Superinfected cows (c6175, c6187, c6188)

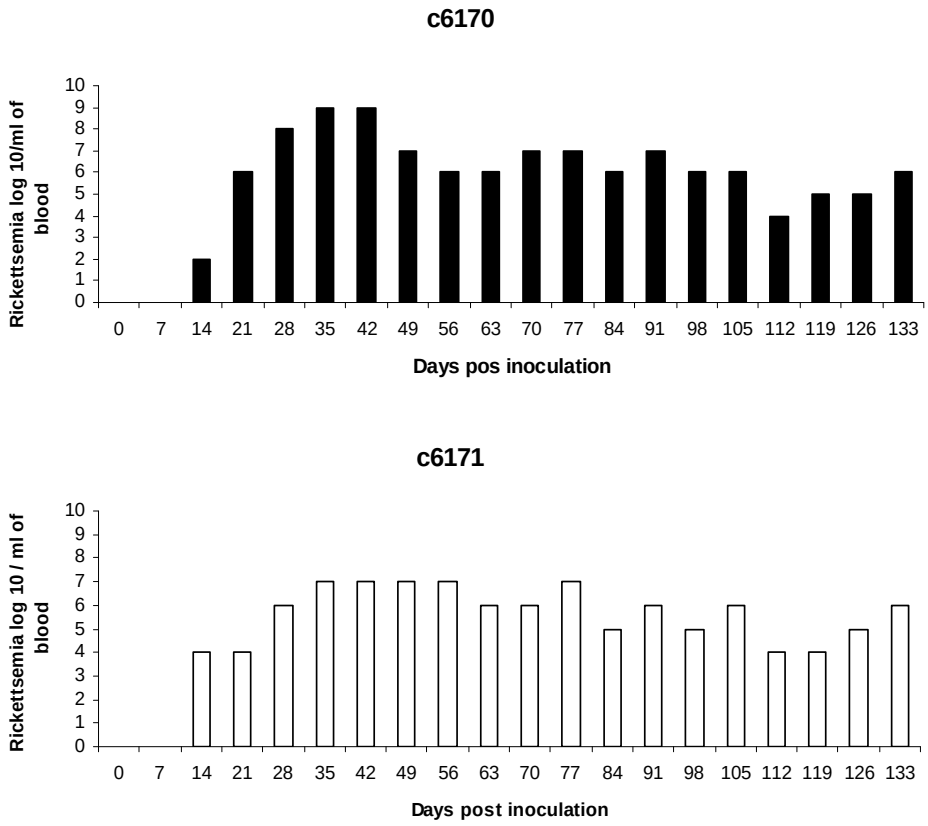

Figure 2 – Single infection control acquisition cows Real Time PCR data analysis. (a) – c6170; (b) – c6171.

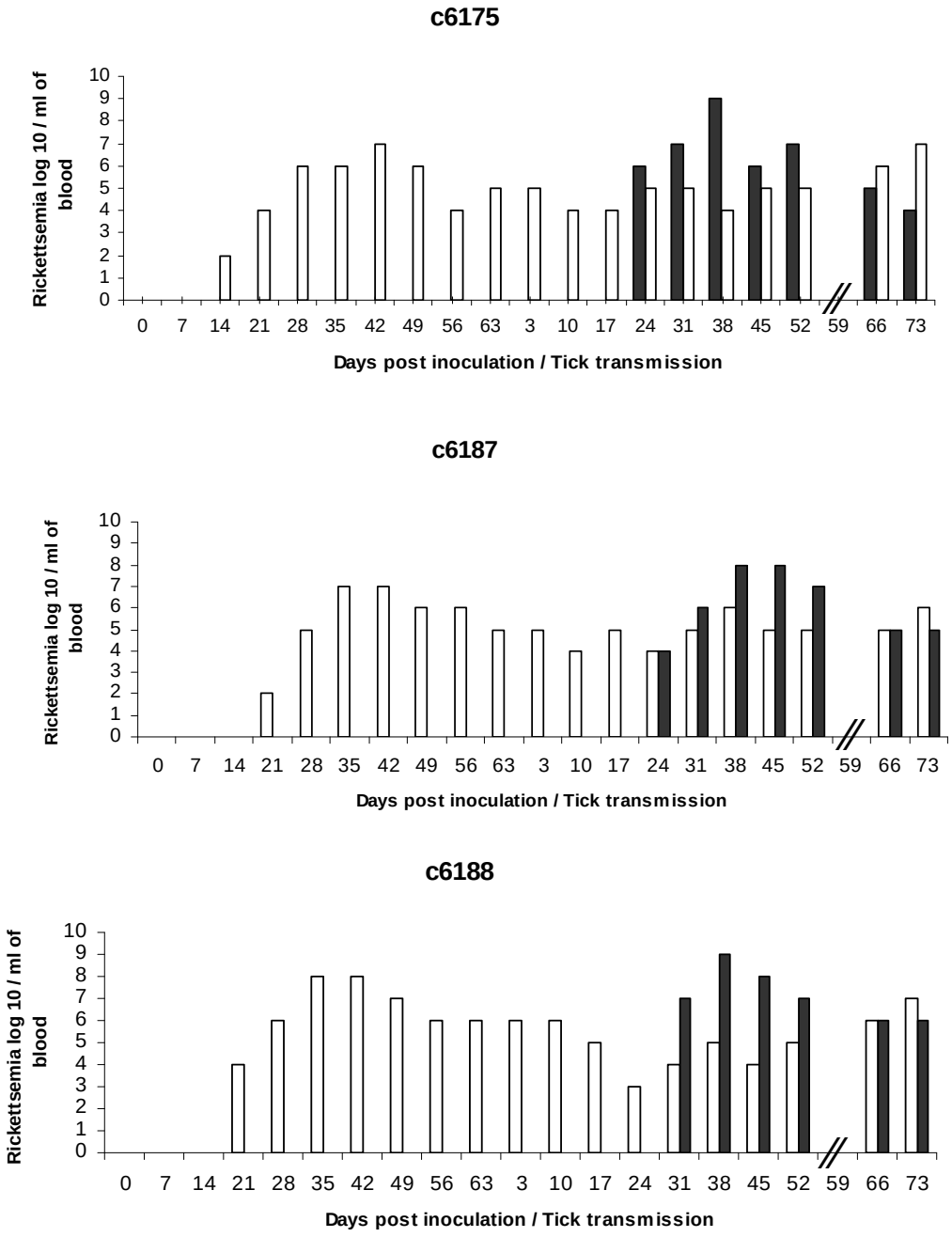

Figure 3 – Superinfected acquisition cows Real Time PCR data analysis. (a) – c6175; (b) – c6187; (c) – c6188.

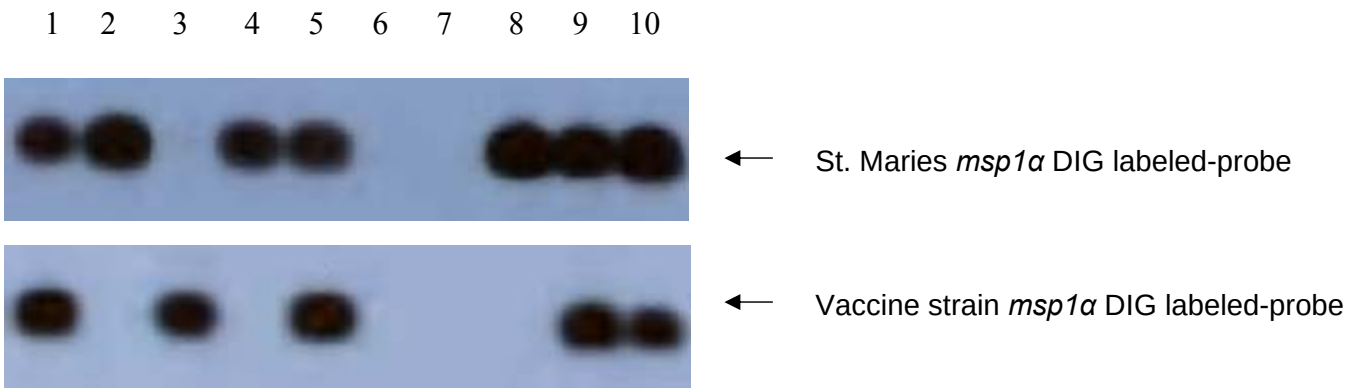

Figure 4 – Southern Blot analysis of tick salivary glands fed on a superinfected animal. Lane 1,5,9 and 10 – co-infection; Lane 6 and7 – no infection; Lane 2,4 and 8 – St. Maries positives; Lane 3 – Vaccine strain positive.

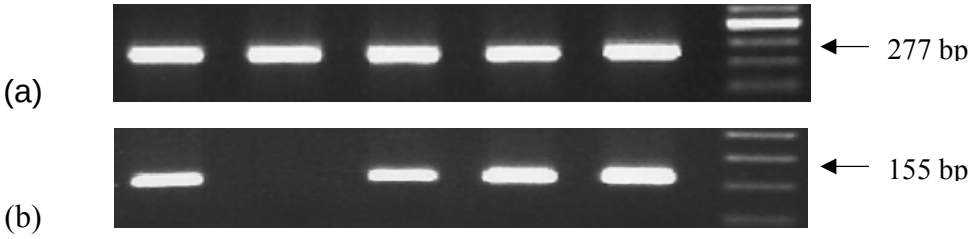

Figure 5 – Confirmation of strain transmission. PCR *msp1α* detection of all five transmission animals. Lane 1 – St. Maries control cow (c32004); Lane 2 – Vaccine strain control cow (c32023); Lanes 3, 4 and 5 – non- superinfected cows (c32001, c31991 and c32008). 4 (a) – DNA Isolation control primers for bovine cytochrome B, amplicon 277bp; 4 (b) – Duplex msp1 primers for vaccine and St. Maries strsins, amplicom 155 bp.

Animal identification number	Parasitemia at week of acquisition (SE mean of parasites/ ml of blood)				
	Controls		Superinfected animals		
	6170 St. Maries	6171 *A. centrale*	6175	6187	6188
A. centrale	-	$10^{6.3\ (\pm 0.4)}$	$10^{6.8\ (\pm 0.6)}$	$10^{7.2\ (\pm 0.05)}$	$10^{7.1\ (\pm 0.4)}$
A. marginale St. Maries	$10^{7(\pm\ 0.1)}$	-	$10^{5.2(\pm\ 0.2)}$	$10^{5.3(\pm\ 0.05)}$	$10^{6(\pm\ 0.05)}$

Table 1 – Real Time PCR rickettsemia during tick acquisition week.

	Southern Blot (% of positives)					Real Time PCR quantification (SE mean no. of parasites/ salivary gland)				
	6170	6171	6175	6187	6188	6170	6171	6175	6187	6188
A. centrale	-	45	42.7	10.2	24.2	-	$10^{2.1(\pm 0.7)}$	$10^{2.4(\pm 0.2)}$	$10^{3.0(\pm 0.4)}$	$10^{2.5(\pm 0.6)}$
St. Maries	79.6	-	18.3	16.9	43.6	$10^{3(\pm 0.9)}$	-	$10^{3.1(\pm 0.71)}$	$10^{3.5(\pm 0.34)}$	$10^{3.0(\pm 0.83)}$
Double positives (Superinfected)	-	-	37.8	63.5	32.2	-	-	$10^{3.7(\pm 1.16)}$	$10^{2.5(\pm 1.19)}$	$10^{2.0(\pm 0.70)}$
						-	-	$10^{2.8(\pm 1.17)}$	$10^{4.0(\pm 1.05)}$	$10^{3.8(\pm 0.51)}$

Table 2 – Southern blot and Real Time quantification on *D. andersoni* salivary glands from acquisition cows (control and superinfected).

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