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Coinfection with Antigenically and Genetically Distinct Virulent Strains of *Babesia bovis* Is Maintained through All Phases of the Parasite Life Cycle▽

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Antigenic polymorphism is a defining characteristic of the *Babesia bovis* variable merozoite surface antigen (VMSA) family. Sequence analysis strongly suggests that recombination between virulent strains contributes to VMSA diversity. While meiosis during the aneuploid stage of the parasite's life cycle in the tick vector *Rhipicephalus* (*Boophilus*) *microplus* is the most probable source of interstrain recombination, there is no definitive evidence that coinfection of the mammalian host or *R. microplus* ticks with more than one virulent strain occurs. Using allele-specific real-time quantitative PCR, we tested the hypotheses that cattle could support coinfection of two antigenically variant virulent tick-transmissible strains of *B. bovis* and that *R. microplus* ticks could acquire and transmit these two divergent strains. The results indicate that both calves and ticks can support virulent *B. bovis* coinfection through all phases of the hemoparasite's life cycle. Neither strain dominated in either the mammalian or invertebrate host, and larval tick progeny, which could be coinfected individually, were also able to transmit both strains, resulting in virulent babesiosis in recipients. While coinfection of the tick vector provides the context in which allelic antigenic diversity can be generated, recombination of VMSA genes could not be confirmed, suggesting that VMSA allelic changes are slow to accumulate.

Antigenic variation and diversity are central to the ability of apicomplexan hemoparasites to break through previously established mammalian host immunity. Rapid antigenic variation, typified by changes in *Plasmodium falciparum var* and *Babesia bovis ves* gene family expression on the surfaces of infected erythrocytes (1, 3, 23), and the apparently slower accumulation of change in merozoite surface proteins both contribute to antigenic diversity (18, 20).

In *B. bovis*, the most virulent cause of the highly prevalent tick-borne disease bovine babesiosis, the multigene variable merozoite surface antigen (VMSA) family (7, 10) plays an important role in the accumulation of surface protein diversity (2, 7, 16, 24). Nucleotide changes present in the *vmsa* genes of multiple *B. bovis* strains and isolates are consistent with intragenic exchange among genetically divergent parasites (2, 7, 12, 15). Homologous recombination during meiosis in the sexual stage of the parasite, which for *B. bovis* occurs in the tick vector *Rhipicephalus* (*Boophilus*) *microplus*, may be responsible for *vmsa* sequence diversity. This would require coinfection of the tick vector by acquisition of a blood meal containing two or more strains. However, little information is available concerning coinfection of virulent tick-transmissible *B. bovis* strains in cattle or tick vectors (13). To begin investigating the mechanism of generating antigenic diversity in *B. bovis*, the dynamics of *B. bovis* coinfection in the natural host and the tick vector needs to be examined.

In this study, we tested the hypotheses that cattle, a natural mammalian reservoir, can support coinfection of virulent tick-transmissible strains and that *R. microplus* ticks can acquire and transmit two genetically and antigenically divergent and virulent strains of *B. bovis*. The kinetics of coinfection in animals simultaneously inoculated with two virulent strains of *B. bovis* and the presence and level of infection in tissues from multiple developmental stages of *R. microplus* ticks after acquisition feeding on coinfected cattle were determined by using allele-specific real-time quantitative PCR (RTQ-PCR). Because the VMSAs appear to play a critical role in vaccine breakthrough, we also examined whether coinfecting strains undergo genetic exchange at the VMSA locus following passage through the tick vector and selection in immune hosts.

MATERIALS AND METHODS

***B. bovis* strains and genomic DNA isolation.** The virulent T2Bo strain of *B. bovis* was originally isolated in southern Texas (19); the L strain is a virulent *B. bovis* strain originally isolated from infected ticks in New South Wales, Australia (17). The T2Bo and L strains are antigenically distinct tick-transmissible strains (11, 17, 19) that have different *vmsa* loci (2). A T2Bo/L strain mixed stabilate containing approximately 5×10^7 infected erythrocytes (for each strain) was prepared by mixing washed and packed erythrocytes with known parasitemia from two splenectomized calves inoculated with either T2Bo strain (c1091) or L strain (c1094) stabilate. Parasitemia in both calves was determined and followed daily using RTQ-PCR (see below). Seven 3- to 6-month-old Holstein calves were purchased from local auction and quarantined for 2 weeks prior to use. For tick acquisition and transmission feeding experiments, calves were splenectomized at the Washington State University Teaching Hospital, Pullman, WA. All animal experiments were approved by the Institutional Animal Care and Use Committee, Washington State University.

Large-scale genomic DNA preparation from phosphate-buffered saline-

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TABLE 1. Oligonucleotide sequences used for RTQ-PCR experiments and amplification of *msa2a/b* and *msa-1*

Oligonucleotide	Application	Sequence (5′–3′)
Lsbp-F	*sbp-1* RTQ-PCR primer	GTACAGTGCTTTCTCTGACATTGATAT
Lsbp-R	*sbp-1* RTQ-PCR primer	CAAAGAAAGTGGAGAACGCATCT
Lsbp-P	*sbp-1* RTQ-PCR probe	AATTACAAACCACACTCCTGACTCA
Tsbp-F	*sbp-1* RTQ-PCR primer	GTACAATGGTTTTTCTGACATGGATAA
Tsbp-R	*sbp-1* RTQ-PCR primer	TCTCAGACACAGCAAACATAGGC
Tsbp-P	*sbp-1* RTQ-PCR probe	ATATTACAGACCACACCCCTGAATC
Nsbp-F	*sbp-1* RTQ-PCR primer	GTCCAAAGAAGTGTATGAACCTTAG
Nsbp-R	*sbp-1* RTQ-PCR primer	GTAGTACATCAGCAATAAAGTCAGCAA
Nsbp-P	*sbp-1* RTQ-PCR probe	CGATGCTAAAGCGATGAAGACAGA
msa-2-F124	*msa-2a/b* PCR primer	CGAAAATGATCGGGAAAATCTTCT
B42/44-R	*msa-2a/b* PCR primer	AAAATGCAGAGAGAACG
msa-1-F1712	*msa-1* PCR primer	GAGTGCGTGTGTGCATATAATTCC
msa-1-R2740	*msa-1* PCR primer	ACAGTCAATCCGCCATAGGTGAA

washed and packed erythrocytes was performed by sodium dodecyl sulfate lysis/proteinase K digestion, followed by phenol-chloroform extraction and a standard ethanol precipitation (22). For small-scale genomic DNA preparations, DNAs were isolated from 0.5 to 1.0 ml of whole blood by guanidine hydrochloride extraction, using a QIAamp DNA Blood Midi kit (QIAGEN, Valencia, CA). Genomic DNA preparation from tick egg masses and individual tick larvae was carried out using a DNeasy tissue kit (QIAGEN) per the manufacturer's instructions.

Tick acquisition and transmission feeding. The La Minita strain of *R. microplus* was used in all tick experiments. Tick acquisition feeding was performed by placing approximately 1 gram of uninfected *R. microplus* larvae (equivalent to approximately 20,000 individual larvae) in skin patches on splenectomized calves (c1084 and c1128) determined to be free of *B. bovis* infection by competitive enzyme-linked immunosorbent assays. Larvae were allowed to molt through the nymphal stage into adults, at which time the calves were inoculated with T2Bo and L strain mixed-infection stabilates containing approximately 5×10^7 infected erythrocytes (for each parasite strain). After adult female tick engorgement, replete females were placed individually into separate incubation wells for ovopositioning at 26°C and 92.5% humidity. *B. bovis* infection levels were determined by light microscopic examination of stained hemolymph smears on day 6 postrepletion. Resulting egg masses from individual replete females were placed individually into sterile containers (one egg mass per container) under the same incubation conditions and allowed to hatch. Subsequent groups of larvae representing progeny from a single female were kept separate from each other and incubated for 3 weeks at 14°C and 92.5% humidity. For tick transmission experiments, 1 gram of larvae pooled from 382 of the infected adults was placed in skin patches on *B. bovis*-free splenectomized calves (c1101 and c1147). Transmission feeding was allowed to continue uninterrupted until clinical signs of babesiosis presented, at which time blood was collected for frozen stabilate preparation, followed by euthanasia of infected animals. Induction of *B. bovis* infective stages within larvae was carried out by placing each group of infected larvae derived from a solitary adult female in a separate skin patch on a *B. bovis*-free calf with an intact spleen (c06). After 72 h of feeding, infected larvae were collected from the patches and placed at −70°C for downstream processing. A two-tailed, pairwise *t* test was used to compare the levels of each strain in calves, egg masses, and larvae. A *P* value of <0.05 was considered significant.

Immunization of calves. To attempt immune selection of antigenically variant parasites after serial tick passage, immunity to both the T2Bo and L strains was generated in calves by coinfection and treatment. Two calves with intact spleens (c4537 and c4569) were inoculated intravenously with the T2Bo/L strain mixed stabilate. The clinical disease course was followed daily by packed cell volume (PCV) and rectal temperature. Animals presenting with severe signs of babesiosis (rectal temperature of >41°C and a PCV of 20%) were symptomatically treated with diminazene diaceturate (1 to 5 mg/kg of body weight) to reduce the severity of clinical disease. Recovery from clinical disease was charted by daily assessment of rectal temperature and PCV. To determine whether the animals had acquired immunity to the T2Bo and L strains, recovered animals were homologously challenged with the same batch of T2Bo/L strain mixed stabilate and similarly followed. Neither animal had an increase in rectal temperature or a drop in PCV for 3 weeks following inoculation, and thus they were considered immune to both strains. Three weeks following the homologous challenge, immunized animals were inoculated intravenously with *B. bovis* stabilates prepared from splenectomized calves that had been infested with larvae coinfected with both the T2Bo and L strains after one (c1101) or two (c1147) serial tick passages.

Allele-specific and nonspecific RTQ-PCR. The copy number of parasites per unit of tissue or whole blood was determined by RTQ-PCR Taqman assays performed on an iQ iCycler and analyzed by iQ software (Bio-Rad, Hercules, CA). Oligonucleotide primer sets were designed to amplify fragments of the gene encoding spherical body protein 1 (*sbp-1*), a single-copy gene that is distinct between Australian and American strains (9). A total of three primer sets with three Taqman probes were used (Table 1). Quantitation of individual strains was carried out using allele-specific oligonucleotide primer sets (L-specific primers Lsbp-F and Lsbp-R and T2Bo-specific primers Tsbp-F and Tsbp-R) in conjunction with allele-specific oligonucleotide probes (L-specific probe Lsbp-P and T2Bo-specific probe Tsbp-P). A non-allele-specific primer set (Nsbp-F and Nsbp-R) with a non-allele-specific oligonucleotide probe (Nsbp-P) that hybridizes to both T2Bo and L *sbp-1* genes was used to screen individual larvae for infection. The positions of the amplification fragments within *sbp-1* were bp 249 to 598 (349 bp) for the L-specific primer set, bp 234 to 445 (211 bp) for the T2Bo-specific primer set, and bp 311 to 607 (296 bp) for the non-allele-specific primer set. All probes were 5′ labeled with Texas Red and 3′ labeled with Black Hole Quencher 2 (Integrated DNA Technologies, Coralville, IA). Twenty-five-microliter amplification reaction mixtures contained 1× iQ Supermix (Bio-Rad) with a 200 nM concentration (each) of forward and reverse primers, 200 nM probe, and 1 μl target DNA. Cycling, following a 3-min 95°C hot start, was as follows: denaturation for 30 seconds at 95°C and annealing/extension for 30 seconds at 61.4°C. Standard curves for the allele-specific primer/probe sets were constructed with reaction mixtures containing 10-fold serially diluted allele-specific plasmids containing a 456-bp fragment of *sbp-1* (ranging from 10 to 1×10^6 or 1×10^7 copies per reaction). Serial 10-fold dilutions of T2Bo *sbp-1* plasmids were used as targets for the construction of non-allele-specific standard curves. The standard curve correlation coefficients for each run ranged from 0.992 to 0.999, with slopes that ranged from −3.398 to −3.357. Triplicate plasmid DNA and experimental DNA samples were run in parallel and placed under identical reaction conditions, except when individual larval samples were analyzed, in which case quintuplet experimental DNA samples were run. The copy number per reaction was determined by calculating the mean of the threshold cycle values for all positive samples plotted against the standard curve.

Amplification and sequencing of *vmsa* (*msa-1* and *msa-2a/b*) genes from genomic DNA. The *msa-2a/b* and *msa-1* genes (less the last 37 bp of the highly conserved 3′ region) (24) of the T2Bo and L strains were amplified from genomic DNA by using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and non-allele-specific primer sets msa-2-F124 plus B42/44-R and msa-1-1712F plus msa-1-2740R, respectively (Table 1). Agarose gel-isolated amplicons were cloned into pCR-4-TOPO (Invitrogen). Miniprep DNA was sequenced using ABI chemistries and analyzed on an ABI3130*xl* genetic analyzer (Applied Biosystems, Foster City, CA).

RESULTS

Allele-specific RTQ-PCR. T2Bo and L strain-specific oligonucleotide primer sets and Taqman probes were designed to amplify and hybridize to allelic fragments of the gene encoding

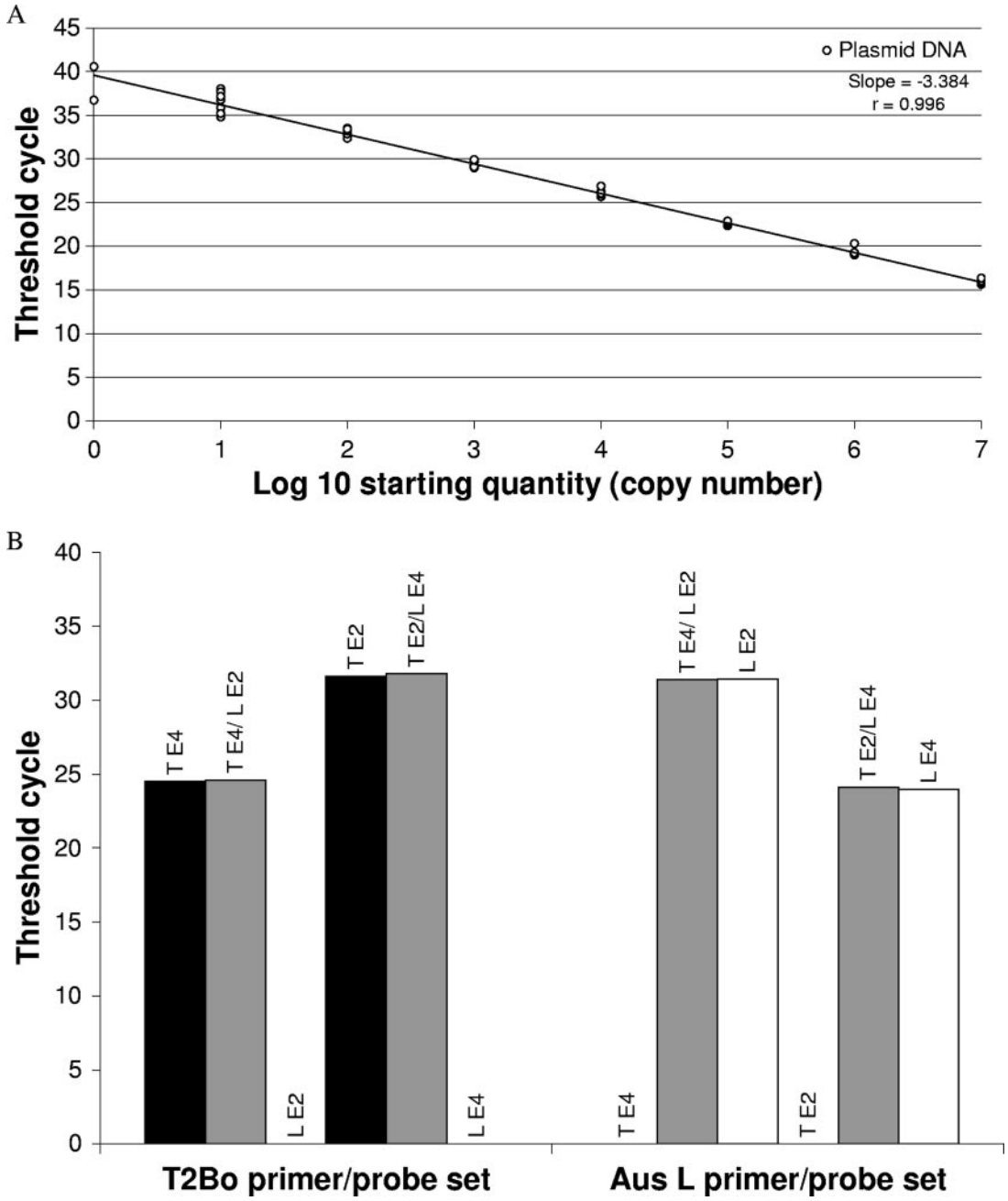

FIG. 1. Evaluation of the sensitivity and specificity of RTQ-PCR allele-specific primer/probe sets. (A) Standard curve generated by T2Bo-specific RTQ-PCR amplification of octuplet samples of T2Bo *sbp-1*-containing plasmids serially diluted from 1×10^7 to 1 copy. *r* is the correlation coefficient. This curve is representative of those generated by the amplification of L *sbp-1*-containing plasmids with the L-specific primer/probe set. (B) RTQ-PCR amplification using T2Bo and L *sbp-1* allele-specific primer/probe sets was performed on individual strains or mixtures of the T2Bo (T) and L (L) strains. E2 and E4 are 1×10^2 and 1×10^4 genome equivalents, respectively. Each data bar represents the mean threshold cycle value per triplicate set of amplification reactions. Target DNA quantities are labeled above each bar. The left half of the graph shows threshold cycle values for amplification reactions carried out using the T2Bo-specific primer/probe set, and the right half of the graph shows threshold cycle values for amplification reactions carried out using the L-specific primer/probe set, as labeled.

spherical body protein 1 (*sbp-1*), a single-copy gene that is divergent between Australian and American *B. bovis* strains (9). The allele-specific primer/probe sets accurately detected plasmid-encoded targets from 1×10^7 to as few as 10 copies per microliter whole blood (Fig. 1A). To verify that the allele-specific primer/probe sets were specific and would perform well in the presence of competing strain DNA, allele-specific amplification reactions with the target genomic DNA with and without the addition of genomic DNA from the other strain were performed. Target genomic DNA quantities used in these experiments were based on preliminary RTQ-PCRs showing that the parasitemia of splenectomized and nonsplenecto-

mized calves acutely infected with these strains ranged from $<1 \times 10^2$ to 1×10^5 parasites per microliter of whole blood. Threshold cycle values for T2Bo-specific reaction mixes containing 1×10^4 or 1×10^2 genome equivalents of the T2Bo strain alone were nearly identical to those for parallel reaction mixes containing the same amount of T2Bo genomic DNA with the addition of either 1×10^2 or 1×10^4 genome equivalents of the L strain, respectively (Fig. 1B). Similarly, threshold cycle values for L-specific amplification reaction mixes containing 1×10^4 or 1×10^2 genome equivalents of the L strain alone were nearly identical to those for parallel reaction mixes containing the same amount of L genomic DNA with either 1×10^2 or 1×10^4 genome equivalents of the T2Bo strain, respectively (Fig. 1B). Moreover, no copies were detected when L genomic DNA was amplified with the T2Bo-specific primer/probe set or when T2Bo genomic DNA was amplified with the L-specific primer/probe set (Fig. 1B). The results indicate that the *sbp-1* allele-specific real-time PCR assays had the necessary sensitivity and specificity for analysis of coinfection experiments.

Quantitative analysis of *B. bovis* coinfection in calves with intact spleens. To determine whether cattle could support coinfection of two virulent strains of *B. bovis*, two calves with intact spleens (c4537 and c4574) were inoculated intravenously with blood stabilates containing approximately 5×10^7 erythrocytes (each) infected with the T2Bo and L strains. Subsequently, allele-specific RTQ-PCR was performed on genomic DNAs isolated from peripheral whole blood collected between 7 and 14 days postinoculation (dpi) (Fig. 2). Both strains were detectable by 9 dpi. The parasitemia for each strain generally rose in parallel during acute infection. While infection levels were significantly different between strains on days 10 to 13, parasitemia on any given day typically stayed within 1 $\log_{10}$ and never differed by more than 2 $\log_{10}$. The parasitemia of both strains was still rising at 13 dpi and ranged from 7.91×10^1 L parasites/μl whole blood in c4537 to as high as 3.03×10^3 T2Bo parasites/μl whole blood in c4574, at which time both calves were treated for clinical signs of babesiosis. During days 9 through 11 for c4537 and days 9 through 12 for c4574, the L strain represented the majority of the parasite population, while on day 13 for both calves, the T2Bo strain predominated. The total parasite loads at 13 dpi were 1.86×10^3 parasites/μl whole blood for c4537 and 3.57×10^3 parasites/μl whole blood for c4574, which are comparable to those in calves with intact spleens inoculated with the T2Bo or L strain alone (8, 27). The results clearly demonstrate that cattle with intact spleens can be coinfected by a blood stabilate with two virulent strains of *B. bovis*. Neither strain consistently predominated the total parasite population, and both reached significant levels capable of causing clinical disease in calves with intact spleens.

Quantitative analyses of *B. bovis* coinfection during tick acquisition, passage, and transmission. To establish whether ticks could become coinfected with two virulent strains of *B. bovis*, *R. microplus* adults were acquisition fed on a splenectomized calf (c1084) inoculated with a stabilate containing approximately equal numbers of both T2Bo and L strain-infected erythrocytes. The quantitative results from the coinfection experiments with spleen-intact animals demonstrated that individual strain parasitemia levels ranged within 2 $\log_{10}$. Because a 2-$\log_{10}$ difference between the coinfecting strain parasitemia

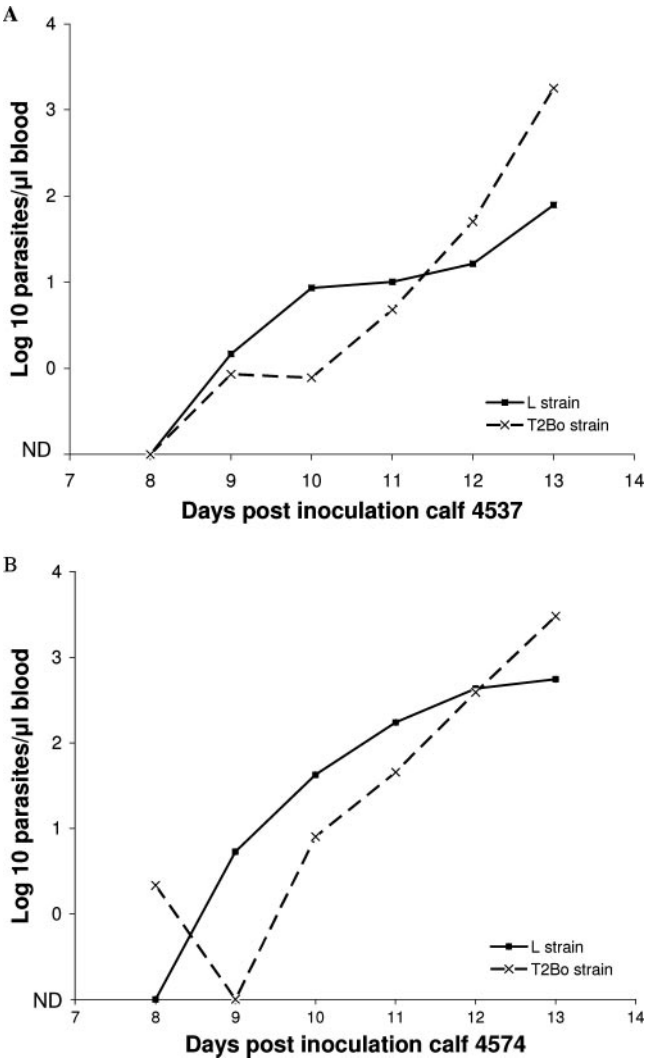

FIG. 2. Allele-specific RTQ-PCR analysis of calves with intact spleens coinfected with two virulent strains of *B. bovis*. T2Bo and L strain parasitemia levels on a $\log_{10}$ scale per microliter of blood from calves c4537 (A) and c4574 (B) were charted against days post-intravenous inoculation with a stabilate containing approximately equal numbers of T2Bo and L strain-infected erythrocytes. ND, nondetectable target. Both calves were treated on day 13 postinoculation for clinical signs of babesiosis. Significant differences in infection level between the two strains occurred at 10 to 13 dpi in both calves ($P < 0.05$).

levels during acquisition feeding could adversely affect the detection of a less dominant strain during tick passage, we followed coinfection in the donor calf to ensure that adult female ticks were exposed to relatively equal numbers of both parasite strains (Fig. 3). Over the entire period of tick acquisition feeding, the ratio of T2Bo/L strain parasitemia in the splenectomized calf ranged from 0.46 to 4.6 at 9 dpi and 14 dpi, respectively, with significant differences in infection levels of the two strains seen only on days 11, 12, and 14. Maximum parasitemia levels were comparable to those in splenectomized animals solely infected with either the T2Bo (11) or L (data not shown) strain.

Twelve hundred engorged female ticks were incubated, and

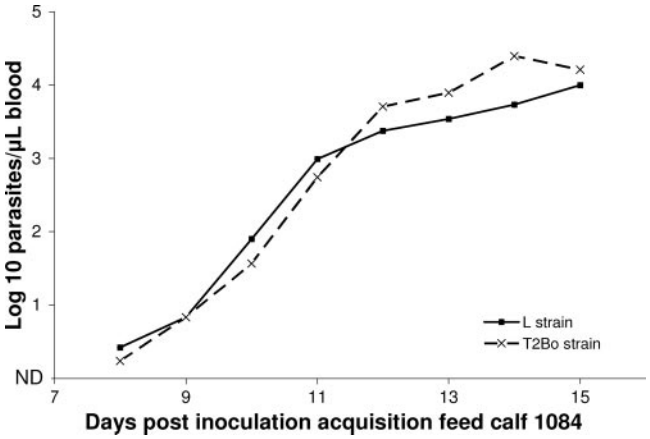

FIG. 3. Allele-specific RTQ-PCR analysis of an acquisition feed splenectomized calf during the time of female adult tick engorgement. T2Bo and L strain parasitemia levels on a $\log_{10}$ scale per microliter of blood from splenectomized calf 1084 are shown for multiple days post-intravenous inoculation with a stabilate containing approximately equal numbers of T2Bo and L strain-infected erythrocytes. ND, nondetectable target. Significant differences in infection level between the two strains occurred at 11, 12, and 14 dpi ($P < 0.05$).

their hemolymph smears were examined to identify those infected with *B. bovis* (62% infection rate). Egg masses from six individually isolated and highly infected adult ticks (more than six kinetes/high-power field) were examined by allele-specific RTQ-PCR to evaluate coinfection of adult female ticks and transovarial cotransmission (Table 2). Both the T2Bo and L strains were detected in each of the egg masses examined, with T2Bo/L strain ratios that ranged from 0.14 to 1.61. In two of six egg masses examined, significantly more Australia L strain parasites than T2Bo parasites were present.

To address whether a single egg could be coinfected, individual larval progeny were investigated by allele-specific RTQ-PCR. Since detectable larval infection rates from parasite-infected *R. microplus* adults can range from 12 to 48% (11), 64 unfed larval progeny from a total of seven infected adults were screened for infection by non-allele-specific RTQ-PCR. Of the 64 larvae, 11 positive individuals derived from seven adult ticks were detected (17% infection rate) and had infection levels comparable to those of larvae infected with T2Bo alone (11). Of these 11 larvae, 7 had parasite levels that were near the detection limit of the assay (<300 parasites/larva) and were not

TABLE 2. *B. bovis* strain-specific infection levels in egg masses from individual replete adult female ticks

Egg mass no.[a]	No. of parasites per µg of *B. bovis*-infected tick genomic DNA		T2Bo/L ratio
	T2Bo strain	L strain	
9.3.1	62.3	75.3	0.83
9.3.2	57.1	46.9	1.22
9.3.3*	60.0	425.9	0.14
9.3.4	103.6	75.4	1.37
10.2.3	232.9	144.7	1.61
10.3.2*	134.3	314.9	0.43

[a] *, infection levels were significantly different between the two strains ($P < 0.05$).

TABLE 3. *B. bovis* strain-specific infection levels in individual larvae

Individual larva no.[a]	No. of parasites per individual larva		T2Bo/L ratio
	T2Bo strain	L strain	
9.1.5.4*	1.18×10^4	1.43×10^4	0.83
9.2.3.7	5.14×10^3	5.26×10^3	0.98
10.1.7.4*	8.40×10^3	1.44×10^4	0.59
10.1.7.7	8.35×10^3	7.30×10^3	1.14

[a] *, infection levels were significantly different between the two strains ($P < 0.05$).

examined further. The remaining four highly infected larvae (>5,000 parasites/larva) were subjected to allele-specific RTQ-PCR (Table 3). In each highly infected larva, both the T2Bo and L strains were present, with T2Bo/L strain ratios that ranged from 0.59 to 1.14. In two larvae, significantly more Australia L strain parasites than T2Bo parasites were present. Sixty larvae placed for 72 h on a calf with an intact spleen to induce *B. bovis* infective stages were similarly examined. However, for the 12 positive larvae identified by nonspecific RTQ-PCR, low-density infections (<300 parasites per larva) precluded accurate quantitative and qualitative differentiation between the coinfecting strains.

To evaluate whether larvae could transmit both coinfecting strains, pooled larval progeny from coinfected adult females were placed on a splenectomized calf (c1101) for transmission feeding, and the resulting parasitemia was analyzed by allele-specific RTQ-PCR (Fig. 4). Parasitemia for both the T2Bo and L strains was detected from day 7 to day 12 post-larval placement, with infection levels that were approximately equal over the entire observation period. The T2Bo/L strain ratio ranged from 0.3 to 2.6 from days 7 to 12, respectively, with an average of 1.28.

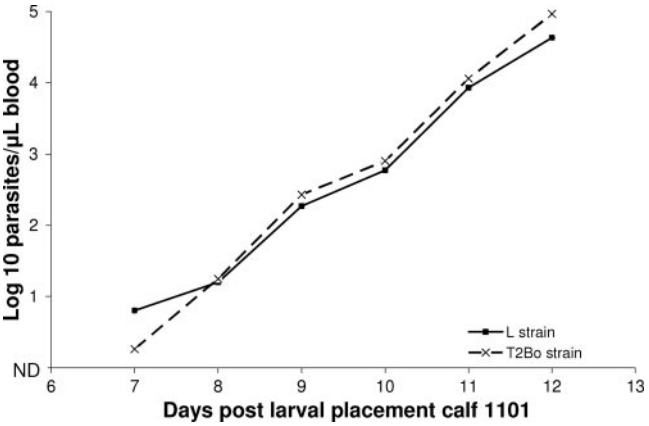

FIG. 4. Allele-specific RTQ-PCR analysis of a transmission feed splenectomized calf. T2Bo and L strain parasitemia levels on a $\log_{10}$ scale per microliter of blood from splenectomized calf 1101 were charted against days postplacement of larvae derived from adult female ticks that had acquisition fed on coinfected calf 1084. ND, nondetectable target. The calf was euthanized on day 12 after the placement of larvae due to clinical signs of babesiosis. Significant differences in infection level between the two strains occurred on days 10 and 12 post-larval placement ($P < 0.05$).

Sequence analysis of *msa-1* and *msa-2a/b* from coinfected, tick-passaged parasites. Since coinfection and cotransmission of two genetically VMSA-distinct strains was clearly possible, we examined if single or multiple tick passages would result in genetic exchange within the VMSA loci. Sequence analysis was carried out on genomic DNAs isolated from splenectomized calves c1101 and c1147, which were used to expand first and second serial tick-passaged parasites, respectively. Both the *msa-1* and *msa-2a/b* genes were PCR amplified from genomic DNA by using non-allele-specific primer sets. No recombinant *msa-1* genes were identified in 91 and 95 clones from c1101 and c1147, respectively, at peak parasitemia. A total of four *msa-2* recombinant sequences, representing four unique genetic exchange events among the *msa-2a/b* genes of the T2Bo and L strains, were detected in a total of 93 and 186 clones sequenced from c1101 and c1147, respectively. Because artifactual recombination between allelic genes can occur during PCR amplification (25), sequence analysis of PCR products from reactions in which the two strains were experimentally mixed and targeted PCR designed to specifically amplify recombinants from experimental genomic DNA were performed. One of 281 clones obtained by PCR with experimentally mixed strain DNAs was a recombinant *msa-2a/b* gene. Two panels of primer sets (18 primer sets for the L/T2Bo recombinant panel and 42 primer sets for the T2Bo/L recombinant panel) targeting potential interstrain recombinants were used to amplify genomic DNA from either c1101 or c1147. Amplification reactions for the experimental calves in all instances either failed to produce amplicons or produced amplicons that were also present in control T2Bo or L genomic DNA reactions. Because variants arising during tick passage, if present, likely represent a small portion of the total population, we attempted to select and enrich for antigenic variants in the first tick-passaged parasites by inoculating them into two calves immunized with both the T2Bo and L strains (c4537 and c4574). No recombinant *msa-2a/b* genes were detected in 186 and 93 clones derived from c4537 and c4574, respectively, at 10 dpi (a day of localized peak parasitemia, as detected by RTQ-PCR [data not shown]). During the 3 weeks following inoculation with the tick-passaged parasites, neither calf showed clinical signs of babesiosis.

DISCUSSION

Sequence analysis suggests that homologous recombination, between or within strains, could account for a significant amount of *B. bovis* allele-encoded VMSA diversity (2, 7, 12, 15). The life cycle of *B. bovis* includes asexual stages within cattle followed by tick acquisition of infected erythrocytes and the subsequent development of the sexual stages within the tick vector *R. microplus* (21). Recombination between strains would most likely arise during the sexual stages within *R. microplus*, which would require tick acquisition of blood meals from coinfected mammalian hosts. While there is evidence that parasites in *B. bovis* carrier cattle are oligoclonal (6, 26), previous attempts to show coinfection with two virulent strains in calves failed to yield a patent detectable infection for both infecting strains (13). In addition, there has been no prior evidence that coinfection of *R. microplus* ticks occurs. Utilizing allele-specific RTQ-PCR, we closely followed the dynamics of acute coinfection with two virulent strains of *B. bovis*. The results indicate that coinfection with virulent strains can readily be established in the natural host and maintained throughout the entire life cycle.

The difference in parasitemia of each infecting strain within calves varied, with any one strain representing as much as 90% or more of the total population. The difference between the two strains, though, rarely exceeded 1 $\log_{10}$. The lack of complete strain dominance by one coinfecting strain over the other presumably improves the probability that individual adult female ticks can acquire more than one genetically distinct parasite population. Clearly, this could differ when coinfection does not occur at the same time or with different coinfecting strains.

Immunity to *B. bovis* in the field is accomplished by the use of live attenuated vaccines. Although these attenuated vaccines generally provide protection against disease, bovine babesiosis in vaccinated animals occurs in association with heterologous isolate infection (4, 5). The origin of these heterologous isolates is unknown, but they could be introduced through infected animal movement. A mixed strain population of parasites in the midgut of replete female ticks, the site of zygote formation, provides fertile ground for the generation of new *B. bovis* variants. These new variants could potentially break through previously established herd immunity at the time of transmission and thus may be the source of heterologous vaccine breakthrough isolates. The potential to generate F1 hybrid and intragenic recombinant variants during the tick stages is supported by our studies demonstrating that all egg masses from the infected adult female ticks examined were uniformly coinfected. Because each individual coinfected tick egg mass was derived from a solitary adult female, we concluded that individual adult female ticks readily support coinfection. Similarly, 100% of highly infected individual larvae examined, by definition derived from individual eggs, were also coinfected. The T2Bo/L strain ratio ranged from 0.14 to 1.61 in egg masses and from 0.59 to 1.14 in highly infected individual larvae. This suggests that the quantitative relationship between the two strains is loosely maintained through tick passage. The effect of large differences in individual coinfecting parasitemia levels, i.e., differences of >2 $\log_{10}$, at the time of acquisition feeding on individual strain population densities within the tick stages could not be evaluated, as the difference in the parasitemia of each coinfecting strain never exceeded 1 $\log_{10}$.

Completion of the *B. bovis* life cycle occurs through transmission of infectious sporozoites from larval salivary glands to the mammalian host. Pooled populations of infected larvae successfully transmitted coinfecting strains to splenectomized calves. The La Minita tick strain and the T2Bo strain were both isolated in southern Texas (19), while the L strain was isolated in Australia (17). Because of their shared geographic niche, one might predict that the T2Bo strain would have an adaptive growth advantage in La Minita ticks compared to the L strain. However, the data do not support this. In two of six egg masses and two of four larvae, there was significantly more L strain present, and the quantitative differences between the two strains at acquisition feeding and, after a several-month passage through ticks, at transmission feeding were maintained. Overall, the results suggest that the La Minita tick strain does not preferentially select between these two geographically distinct strains of *B. bovis* at a biologically significant level. One

possible explanation for this is that receptors used by *B. bovis* to enter tick cells may be in excess, precluding receptor competition between the two strains, or that both strains tested bind the receptors equally well. Tick receptors used by *B. bovis* during its passage through the tick have not been identified.

The *B. bovis* VMSA family, expressed from multiple distinct alleles, plays an important role in antigenic diversity. Significant amounts of sequence variation in the members of this family have been closely associated with populations of parasites that cause clinical disease in vaccinated animals (2, 16). Sequence analyses of the *vmsa* genes suggested that intergenomic genetic exchange occurring between heterologous isolates contributes to this variation (2, 7, 16). In this study, sequence analysis of *msa-2a/b* clones from transmission calves demonstrated only four recombinant clones through two tick passages. The prevalence of recombinant clones, though, was four times greater in the amplified genomic DNA from the transmission calves (4/279 clones) than in our experimentally mixed DNA (1/281 clones), suggesting that a portion of the sequence changes may represent recombination events taking place during tick passage. Nevertheless, because panels of PCRs targeted to specifically amplify recombinants were negative, we could not conclusively determine that all recombinants were not artifacts of PCR. All sequenced *msa-1* clones were identical to the parental coinfecting strains. To enrich for *vmsa* recombinant parasites, we inoculated animals immunized by T2Bo/L strain coinfection and immune to both T2Bo and L strains (data not shown) with a first-tick-passage stabilate. These immunized animals did not present with signs of clinical babesiosis, and no *msa-1* or *msa-2a/b* recombinants were identified.

The existence of *B. bovis* variants in tick-passaged parasites that have recombinant *vmsa* genes cannot be excluded. Based on the number of clones examined, these variants, if present, would represent less than 3% of the population with 95% confidence (28). These findings are not necessarily unexpected. First, despite general nucleotide sequence conservation in the VMSA intergenic regions (data not shown), the two strains used in these experiments may be genetically incompatible for recombination to take place at the VMSA locus or over the entire genome. Second, the widely spaced time intervals observed between outbreak presentations of vaccine breakthrough heterologous isolates (4) suggests that the generation of significantly divergent parasite populations capable of breaking through immunity in endemic regions requires numerous tick passages. Lastly, it is likely that immune system evasion requires the acquisition of antigenic variation in multiple proteins, not just the VMSAs. If this is correct, the stringent immune selection system generated by premunition in these experiments may have prevented the expansion of VMSA recombinant variants to a level necessary for detection without an accumulation of other antigenic changes necessary for immune evasion. The production of diverse variants with multiple recombination sites was recently shown for *Theileria parva* passaged through its tick vector, *Rhipicephalus appendiculatus* (14). Genetic exchange occurring outside the *vmsa* locus has yet to be evaluated. Along with the assessment of overall population heterogeneity induced by tick transmission, this is the focus of our current investigations.

In summary, the results of these experiments indicate that calves with intact spleens can readily support coinfection of two highly divergent and virulent populations of *B. bovis* and that individual *R. microplus* ticks can acquire and their larval progeny can transmit these strains of *B. bovis*. Sequence analyses of *msa-1* and *msa-2a/b* genes from tick-passaged parasite populations indicate that two serial tick vector and mammalian host passages do not result in detectable numbers of intragenic *vmsa* variants or generate parasites capable of breaking through parental strain immunity to cause clinical disease. The findings taken together suggest that although the tick vector provides a stage on which genetic exchange among distinct virulent parasite populations can take place, numerous tick passages are likely required to introduce enough composite antigenic variation to effect escape from the mammalian host adaptive immune response.

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