Quantitative Differences in Salivary Pathogen Load during Tick Transmission Underlie Strain-Specific Variation in Transmission Efficiency of *Anaplasma marginale*[▽]

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The relative fitness of arthropod-borne pathogens within the vector can be a major determinant of pathogen prevalence within the mammalian host population. Strains of the tick-borne rickettsia *Anaplasma marginale* differ markedly in transmission efficiency, with a consequent impact on pathogen strain structure. We have identified two *A. marginale* strains with significant differences in the transmission phenotype that is effected following infection of the salivary gland. We have proposed competing hypotheses to explain the phenotypes: (i) both strains are secreted equally, but there is an intrinsic difference in infectivity for the mammalian host, or (ii) one strain is secreted at a significantly higher level and thus represents delivery of a greater pathogen dose. Quantitative analysis of pathogen replication and secretion revealed that the high-efficiency St. Maries strain replicated to a 10-fold-higher titer and that a significantly greater percentage of infected ticks secreted *A. marginale* into the saliva and did so at a significantly higher level than for the low-efficiency Israel vaccine strain. Furthermore, the transmission phenotype of the vaccine strain could be restored to that of the St. Maries strain simply by increasing the delivered pathogen dose, either by direct inoculation of salivary gland organisms or by increasing the number of ticks during transmission feeding. We identified morphological differences in the colonization of each strain within the salivary glands and propose that these reflect strain-specific differences in replication and secretion pathways linked to the vector-pathogen interaction.

The predominance of a specific pathogen strain in the host population reflects its overall fitness advantage and is a major determinant of the consequent disease pattern (7, 17). We have investigated the strain structure of the tick-borne bacterium *Anaplasma marginale* in its natural reservoir hosts, domestic and wild ruminants, and identified a predominance of specific strains within spatially and temporally defined reservoir host populations (4, 12, 17, 19). We hypothesize that strain predominance is determined by the strain-specific transmission fitness within the tick vector. This overall hypothesis is supported by identification of genetically distinct *A. marginale* strains with marked differences in transmissibility (24). However, the basis for these strain-specific differences in transmissibility is poorly understood.

During tick acquisition feeding on an infected reservoir host, *A. marginale* enters the midgut epithelium and undergoes initial replication before transiting to tick salivary glands and invading the epithelial cells (6, 24). Within the salivary gland epithelial cells, *A. marginale* undergoes a second round of replication, and it is secreted into the saliva concomitant with tick transmission feeding on a new mammalian host (6, 12, 24). Accordingly, both the midgut and salivary gland have been

identified as sites where transmission differences among *A. marginale* strains are manifested (3, 21, 24). At the level of the midgut, it is unclear whether specific strains differ in their ability to enter the midgut epithelial cells or whether the difference is in subsequent replication. In contrast, within the salivary gland epithelium, there is a specific transmission phenotype that occurs postinvasion (12, 24). Highly efficiently transmitted strains replicate to 10^6 to 10^7 organisms per salivary glands and, as shown using the St. Maries strain of *A. marginale*, can be consistently transmitted to naïve animals by feeding fewer than 10 infected *Dermacentor andersoni* ticks (5, 18, 20). Interestingly, the Israel vaccine strain (*A. marginale* subsp. *centrale*) also invades the salivary glands but is not transmitted using cohorts of 100 ticks (24).

We propose two alternative hypotheses to explain the different transmission phenotypes represented by the *A. marginale* St. Maries and the Israel vaccine strains. The first is that there is a decrease in replication of the vaccine strain within the tick vector and/or a reduced secretion into the saliva and thus insufficient organisms to exceed the minimal infective dose for transmission. If this is correct, then the infection threshold could be reached by simply increasing the number of transmission-feeding ticks to achieve the same level of organisms secreted by the highly efficient St. Maries strain of *A. marginale*. Alternatively, the second hypothesis is that there is an intrinsic decreased infectivity of the vaccine strain during a passage within the tick vector, and thus reaching the threshold would require secretion of a significantly greater number of

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TABLE 1. *Anaplasma* strain-specific infection rates and levels in *Dermacentor andersoni*

Strain and feeding	Midgut		Salivary glands	
	Infection rate[a] (no. positive/no. tested)	Infection level[b] (mean ± SD)	Infection rate (no. positive/no. tested)	Infection level (mean ± SD)
St. Maries				
Acquisition[c]	100 (25/25)	$10^{6.4 \pm 0.45}$	100 (25/25)	$10^{5.3 \pm 0.76}$
Transmission[d]	100 (25/25)	$10^{6.7 \pm 0.61}$	100 (25/25)	$10^{7.4 \pm 0.68}$
Israel vaccine				
Acquisition[c]	92 (23/25)	$10^{5.3 \pm 0.59}$	88 (22/25)	$10^{4.5 \pm 0.77}$
Transmission[d]	92 (23/25)	$10^{5.2 \pm 0.87}$	88 (22/25)	$10^{6.1 \pm 0.89}$

[a] Percentage of fed ticks that acquired infection.
[b] Number of bacteria per midgut or salivary gland pair.
[c] Ticks were acquisition fed on calves infected with each specific strain.
[d] Ticks were transmission fed on naïve (competitive enzyme-linked immunosorbent assay-seronegative, *msp5* PCR-negative) calves.

organisms of the vaccine strain than of the St. Maries strain. Here we describe the testing of these hypotheses and present the results in context of vector-pathogen interactions that affect the pathogen strain structure in the mammalian reservoir host population.

MATERIALS AND METHODS

Infection of ticks and pathogen replication within tick salivary glands. The specific-pathogen-free Reynolds Creek colony of *Dermacentor andersoni* and the St. Maries and the Israel vaccine strains used in these experiments have been described in detail previously (6, 21, 24). Adult male *D. andersoni* ticks were allowed to acquisition feed for 7 days on calves infected with either the St. Maries or the Israel vaccine strain. Following an additional 7 days of incubation at 26°C to allow complete digestion of the blood meal and eliminate any possibility of mechanical transmission, ticks were then transmission fed on naïve (competitive enzyme-linked immunosorbent assay-seronegative and *msp5* PCR-negative) age- and gender-matched Holstein calves (12, 24). Cohorts of both acquisition-fed and transmission-fed ticks were dissected and midgut and salivary glands isolated from individual ticks for determination of infection rate (percentage of fed ticks that acquired infection) and infection level (bacterial numbers in each tissue). The infection rate was determined by *msp5* PCR amplification, and organisms were quantified to determine infection level using real-time PCR as previously described in detail for both strains (6, 24).

Pathogen localization within salivary glands. The presence of each strain in the granular acinar cells of the salivary glands was examined using immunohistochemistry, followed by subcellular localization using transmission electron microscopy. For immunohistochemistry, transmission-fed ticks were fixed in 10% formalin and embedded in paraffin, and sequential 4-μm sections were deparaffinized in Clear-Rite and then hydrated in an ethanol gradient. Sections were treated with citrate solution (pH 6) (Zymed, Carlsbad, CA) for antigen retrieval and steamed for 20 min as previously described (6, 21, 23). The sections were stained using 2 μg/ml of monoclonal antibodies specific for each strain. Antibody ANAR49 binds the St. Maries strain, and its use in immunohistochemical detection of this strain has been previously reported (21). To develop a monoclonal antibody reactive with the Israel vaccine strain, mice were immunized with organisms isolated from infected erythrocytes and hybridomas generated using standard procedures as previously described for *A. marginale* (14, 16). Hybridoma supernatants were screened for reactivity with the vaccine strain by immunoblotting and by immunohistochemistry. Monoclonal antibody 132/704.13 was identified as reactive with the vaccine strain in both assays, and supernatants from twice-cloned hybridomas were used for immunohistochemistry on infected ticks (21). Following the primary antibodies, ANAR49 for the St. Maries strain and 132/704.13 for the vaccine strain, horseradish peroxidase-labeled anti-mouse immunoglobulin (Dako Corp., Carpinteria, CA) and 3-amino-9-ethylcarbazole containing hydrogen peroxide were used to detect binding. Sections were counterstained with Mayer's hematoxylin (6, 21). Monoclonal antibodies against *Trypanosoma brucei* (2 μg/ml) were used as a negative control on sequential sections of the same ticks exposed either to the St. Maries strain or the Israel vaccine strain.

For subcellular localization of bacteria, salivary glands were dissected and fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer

at 4°C (10, 11). Tissues were rinsed in 0.1 M cacodylate buffer, postfixed in 2% OsO₄ for 2 h at room temperature, and then rinsed in cacodylate buffer. Following dehydration in an ethanol gradient, samples were infiltrated with acetone and embedded in Spurr's resin. Thin sections (90 nm) were placed on nickel grids and stained in 4% uranyl acetate for 10 min and in Reynolds lead for 3 min. Sections were examined on a JEOL JEM 1200 EX transmission electron microscope.

Pathogen secretion. To quantify the salivary secretion of *A. marginale*, salivation was induced in a cohort of infected, transmission-fed ticks. Briefly, approximately 10 μl of dopamine hydrochloride (100 mg/ml in a 1.2% saline solution) was inoculated into the membrane surrounding the base of the coxa of the fourth leg of individual ticks using a 12.7- by 0.21-mm needle (8, 22). Saliva was collected directly from the mouth parts during a 20-min period and the total collected volume immediately placed into 50 μl cell lysis solution (Qiagen Inc., Valencia, CA) with proteinase K (2 μg/ml) and incubated at 56°C overnight. Following incubation, dilution with 450 μl of cell lysis solution with glycogen (70 μg/ml), and removal of proteins, genomic DNA was precipitated in 100% isopropanol, washed in 70% ethanol, and resuspended in 30 μl of hydration solution (Qiagen Inc., Valencia, CA). *A. marginale*-positive saliva samples were identified by using *msp5* PCR and the bacteria quantified in positive samples using real-time PCR (24).

Strain-specific quantitative transmission. Based on the observed levels of replication and salivary secretion for each strain, two approaches were used to determine if these quantitative differences between strains accounted for the phenotypic differences in transmissibility. For the first, salivary glands were dissected from transmission-fed ticks, and homogenates were prepared in RPMI 1640 medium and inoculated intravenously into splenectomized, naïve calves (9). The number of salivary glands used was determined by the differences in organism levels in the salivary glands between the two strains as quantified by real-time PCR. For the second approach, the number of transmission-feeding ticks infected with the vaccine strain was increased, based on the tick infection rate and levels in saliva, to approximate a similar delivery inoculum represented by feeding 10 ticks infected with the St. Maries strain. The ability of ≤10 Reynolds Creek colony *D. andersoni* adult males to transmit the St. Maries strain has been replicated and reported previously (5, 18, 20, 24). Following either salivary gland homogenate inoculation or tick feeding, calves were monitored by microscopic examination of Giemsa-stained blood smears and infection was confirmed by *msp5* PCR (24). The strain identity was confirmed by *msp5* amplicon sequencing and alignment with the strain-specific sequences previously reported for these two strains (1, 15).

RESULTS

Infection of ticks and pathogen replication within tick salivary glands. Ticks were acquisition fed on calves infected with either the St. Maries or the Israel vaccine strain. During the 7-day acquisition-feeding period, the mean bacteremia levels were 10^8 organisms per ml of blood for each strain, as determined by *msp5* real-time PCR (data not shown). The tick infection rate for the St. Maries strain following acquisition feeding was 100% (Table 1), with mean levels of $10^{6.4 \pm 0.45}$ per
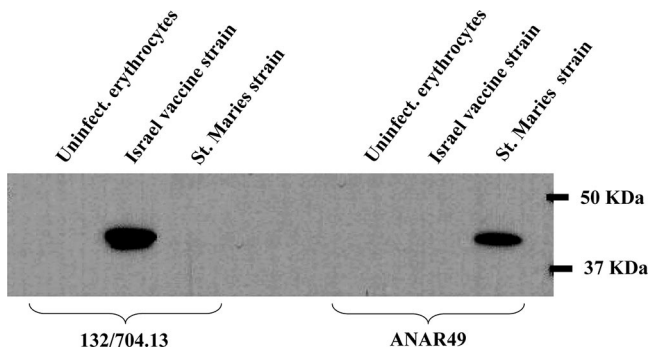
FIG. 1. Reactivity of strain-specific monoclonal antibodies. 132/704.13, antibody reactive with the Israel vaccine strain; ANAR49, antibody reactive with the St. Maries strain. Uninfect. erythrocytes, uninfected bovine erythrocytes as negative control. The positions of the molecular size markers are indicated on the right.

midgut and $10^{5.3 \pm 0.76}$ per salivary gland pair. During transmission feeding, further replication within the salivary glands increased the levels of the St. Maries strain 100 times (Table 1). The tick infection rate for the Israel vaccine strain was

similar at approximately 90%; however, the levels in both midgut and salivary glands were decreased 10-fold compared to those of the St. Maries strain (Table 1). Similar to the case for the St. Maries strain, the levels of the vaccine strain increased 100 times during transmission feeding but remained at reduced levels compared to those of the St. Maries strain (Table 1).

Pathogen localization within salivary glands. The specificity of the monoclonal antibodies was confirmed by immunoblotting using both strains (Fig. 1). These antibodies were then used to localize bacteria within the salivary glands of transmission-fed ticks using immunohistochemistry. Both strains colonized the granular acini of the salivary gland (Fig. 2). However, the St. Maries strain consistently developed multiple colonies within the acini, while the vaccine strain formed only large single colonies (Fig. 2). There was no reactivity of either strain with the negative control monoclonal antibody against *Trypanosoma brucei* (Tryp1E1) on sequential sections of the same infected ticks (Fig. 2).

This same pattern of infection within granular acinar cells was confirmed by transmission electron microscopy. The Israel
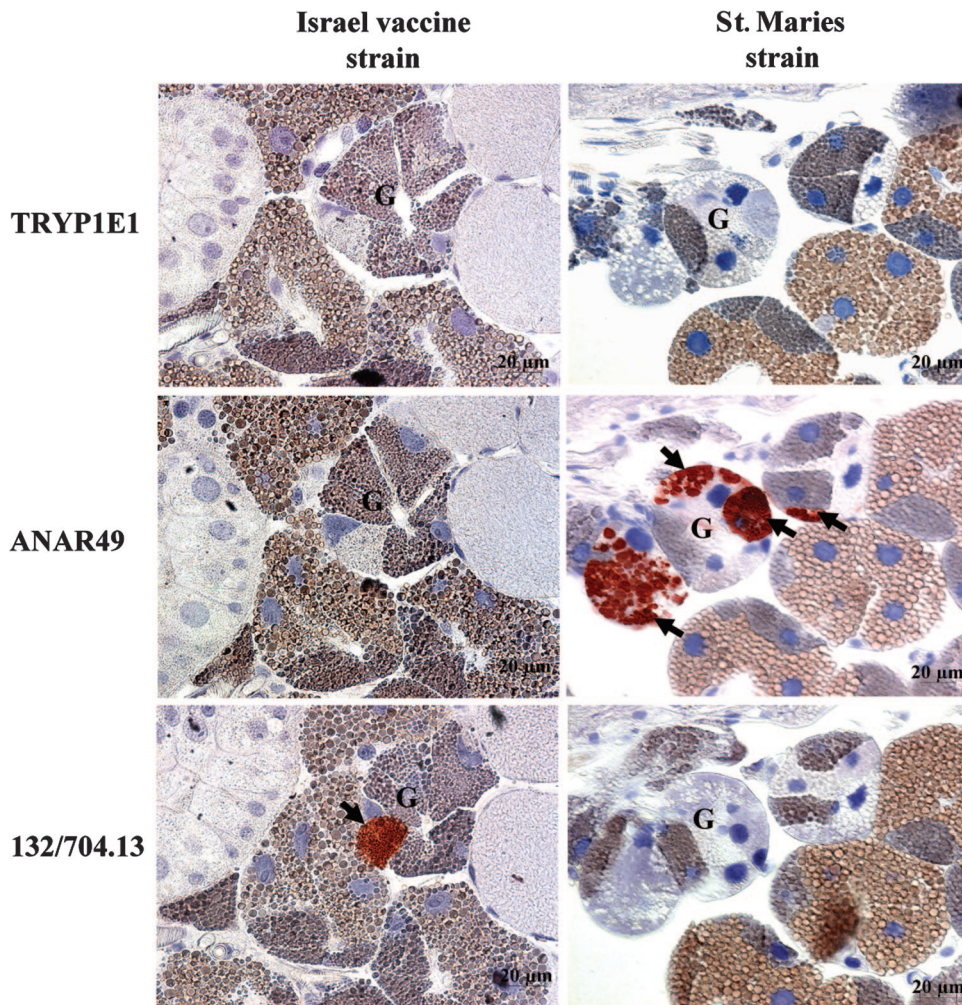

FIG. 2. Localization of *Anaplasma* colonies (arrows) within the granular acinar cells (G) of *Dermacentor andersoni* salivary glands. TRYP1E1, isotype matched control monoclonal antibody reactive with *Trypanosoma brucei*; ANAR49, antibody binding the St. Maries strain; 132/704.13, antibody binding the Israel vaccine strain.
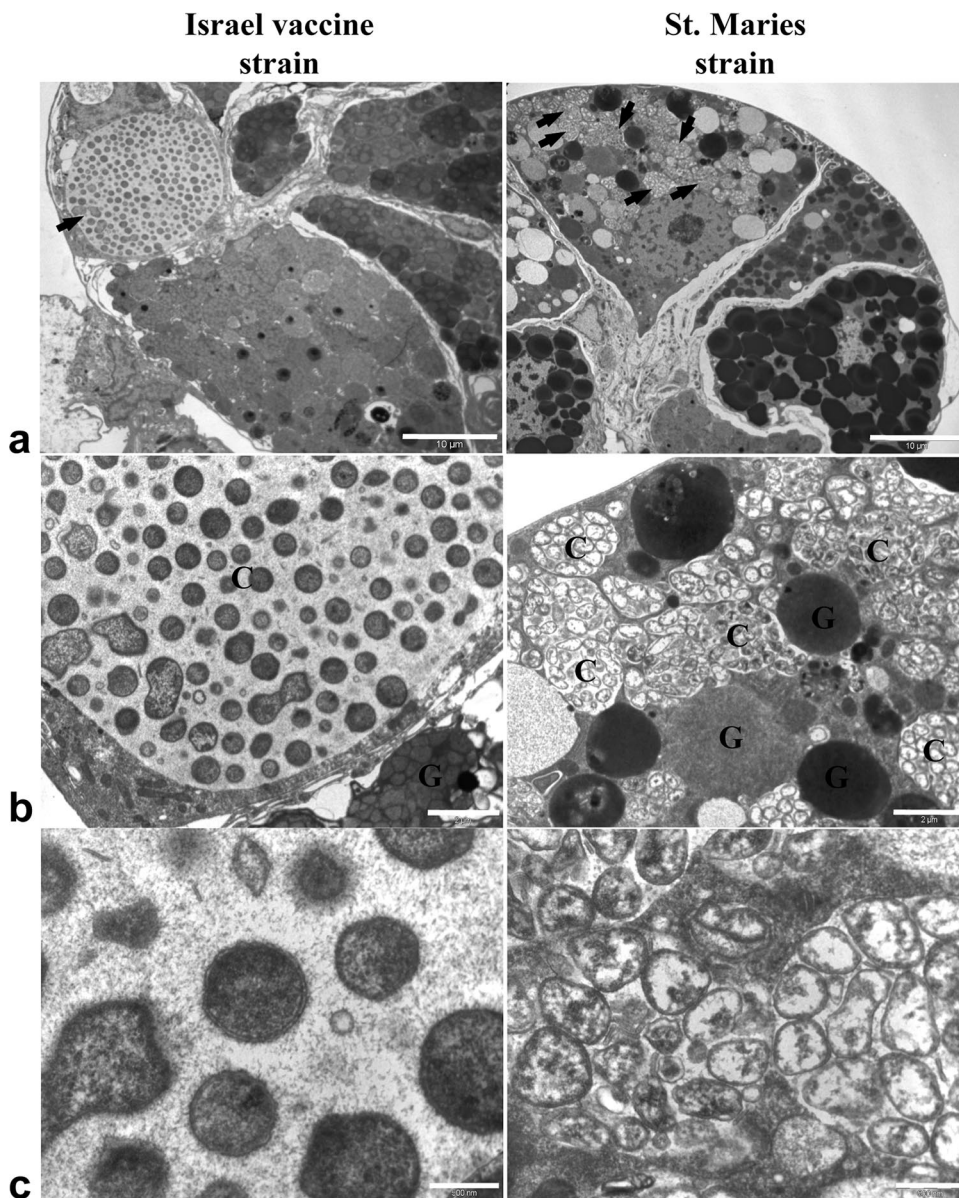
Israel vaccine strain

St. Maries strain

FIG. 3. Transmission electron micrographs of *Anaplasma* colonies within tick salivary glands. (a) Granular acinar cells of salivary glands containing *Anaplasma* colonies. Bar, 10 μm; magnification, ×2,000. Arrows indicate the colonies. (b) Single vaccine strain colony and multiple St. Maries strain colonies within the salivary glands. Bar, 2 μm; magnification, ×10,000. C, *Anaplasma* colonies; G, granules of the acinar cells. (c) Individual organisms within *Anaplasma* colonies. Bar, 0.5 μm; magnification, ×40,000.

vaccine strain formed large single colonies with relatively low numbers of electron-dense organisms, characteristic of the infectious state, within the colonies, while the St. Maries strain developed multiple colonies composed of closely packed electron-lucent organisms, characteristic of the replicative state (11), and were consistently located adjacent to acinar granules (Fig. 3). Salivary glands from ticks of the same colony fed identically but on uninfected animals did not, as expected, contain bacterial colonies (data not shown).

Pathogen secretion. Saliva from transmission-fed ticks infected with the St. Maries strain contained a mean level of $10^{4.1}$ *A. marginale* organisms per μl, and >90% of the individual ticks were positive (Table 2). In contrast, only 30% of the individual

ticks were saliva positive for the vaccine strain, and the levels were 10-fold lower than those of the St. Maries strain (Table 2).

Strain-specific quantitative transmission. Calves ($n = 2$) were inoculated with salivary gland homogenates prepared from 10 ticks infected with the St. Maries strain. This inoculum contained $10^{8.4}$ organisms of the St. Maries strain of *A. marginale*. Both calves were infected and progressed to develop acute high-level bacteremia ($\geq 10^8$ *Anaplasma* organisms/ml of blood) as determined by microscopic examination of Giemsa-stained blood smears. The identity of the St. Maries strain was confirmed by amplification of *msp5* (Fig. 4) followed by sequencing the amplicon to identify the strain-specific sequence. Calves were identically inoculated with salivary gland homog-

TABLE 2. Quantification of *Anaplasma* in saliva of transmission-fed *Dermacentor andersoni*[a]

Strain	Salivary glands		Saliva	
	Infection rate[b] (no. positive/ no. tested)	Infection level[c] (mean ± SD)	Infection rate[d] (no. positive/ no. tested)	Infection level[e] (mean ± SD)
St. Maries	100 (25/25)	$10^{7.5 \pm 0.62}$	96 (24/25)	$10^{4.1 \pm 0.86}$
Israel vaccine	92 (46/50)	$10^{6.5 \pm 0.69}$	30 (15/50)	$10^{3.1 \pm 0.49}$

[a] The salivary glands and saliva represent the same cohort of ticks.
[b] Percentage of fed ticks that were infected.
[c] Number of bacteria per salivary gland pair.
[d] Percentage of fed ticks that secreted bacteria in the saliva.
[e] Number of bacteria per μl of saliva.

enates of ticks infected with the Israel vaccine strain using two different inoculum sizes: inoculation with homogenates of 15 ticks representing $10^{7.2}$ bacteria ($n = 2$ calves) and inoculation with homogenates of 150 ticks representing $10^{8.2}$ bacteria ($n = 2$ calves). Both sets of calves became infected and progressed to develop acute high-level bacteremia ($\geq 10^8$ *Anaplasma* organisms/ml of blood). The identity of the vaccine strain was confirmed by amplification of *msp5* (Fig. 4) followed by sequencing the amplicon to identify the strain-specific sequence. Having demonstrated that the vaccine strain organisms in the salivary gland were infectious, we tested whether increasing the number of ticks infected with the vaccine strain to approximate the saliva levels represented by 10 St. Maries strain-infected ticks would result in vaccine strain transmission. As both the percentage of ticks with organisms in saliva and the number of organisms per μl of saliva were decreased for the vaccine strain compared to the St. Maries strain, a >35-fold increase in the number of vaccine strain-infected ticks was predicted to be sufficient for successful transmission. A total of 425 adult male *D. andersoni* ticks were transmission fed for 7 days on a naïve calf. Bacteremia was detected microscopically, and the identity of the vaccine strain was confirmed by sequencing the *msp5* PCR amplicon (data not shown). As a positive control, the St. Maries strain was transmitted to a separate naïve calf at the same time.

DISCUSSION

The St. Maries strain is a prototypically high-transmission-efficiency strain with consistent transmission in multiple replicate trials to naïve calves using 10 infected adult male *D. andersoni* ticks (5, 24). Consistent with this phenotype, trials using one and three infected ticks have also resulted in trans-

mission of the St. Maries strain (18, 20). In contrast, replicate trials using 100 adult male *D. andersoni* ticks, which were acquisition fed during either the acute (24) or persistent (M. F. B. M. Galletti, unpublished data) phase of infection, have failed to transmit the Israel vaccine strain. The present study indicates that the low-transmission-efficiency phenotype of the Israel vaccine strain in *D. andersoni* largely reflects delivery of a diminished dose during tick feeding compared to the St. Maries strain. This diminished dose reflects a significantly lower level within the tick vector, fewer ticks secreting organisms in the saliva, and a lower number of organisms secreted into the saliva. Although we have shown that both strains are infectious when directly inoculated into susceptible animals, in the absence of 50% infectious dose determination for both strains, we cannot conclude that the two strains have equal intrinsic infectivity. Thus, diminished intrinsic infectivity may also contribute to the low-efficiency transmission phenotype of the vaccine strain. Nonetheless, the primary determinant of transmission efficiency appears to be the pathogen dose in the saliva.

A quantitative basis for transmission efficiency phenotypes has consequences for our understanding of transmission both epidemiologically and mechanistically at the level of the pathogen-vector interaction. *A. marginale* strains have previously been reported as "non-tick transmissible," consistently raising the question as to how these strains were propagated in the field, given the very low efficiency of mechanical transmission (3, 24). A quantitative basis for transmission efficiency phenotype rather than a binary function (a strain is or is not tick transmissible) provides an explanation of how low-efficiency strains can be transmitted but only under conditions of very high tick burden. The *D. andersoni* tick burden on cattle under natural conditions is normally low and thus would favor strains with high transmission efficiencies (26). This is supported by the predominance of the EMΦ strain, a strain consistently transmitted using ≤10 ticks, within a host reservoir population under conditions of natural transmission (5). However, tick burden can increase dramatically based on shifts in climate and land use, resulting in episodic high tick burdens that could allow transmission of low-efficiency strains.

Mechanistically, the strain-specific quantitative differences in replication and secretion expand the search for the pathogen determinants of transmission efficiency. Previous investigation has focused primarily, if not solely, on *A. marginale* surface molecules, with the presumption that successful infection of either midgut epithelial cells or salivary gland epithelial cells was the primary determinant of transmissibility (2, 3, 13). Our
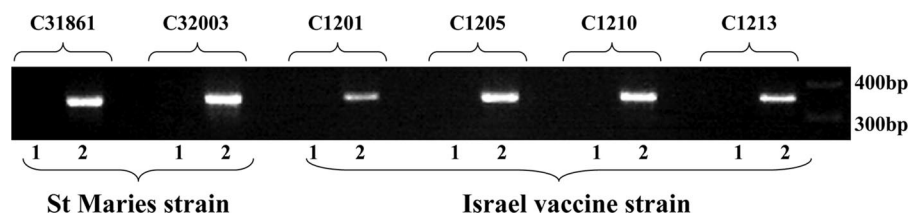

FIG. 4. Transmission by inoculation of salivary gland *Anaplasma* homogenates. Calves were inoculated with homogenates containing $10^{8.4}$ St. Maries strain organisms (C31861 and C32003), $10^{7.2}$ vaccine strain organisms (C1201 and C1205), or $10^{8.2}$ vaccine strain organisms (C1210 and C1213). PCR amplification of *msp5* from preinoculation blood (lane 1) or during acute bacteremia (lane 2) is shown. The positions of the molecular size markers are indicated on the right.

findings indicate that infection of the salivary gland epithelium is not the key determinant. While both strains invade and colonize granular acinar cells within the tick salivary gland, there are clear morphological differences in colony structure. The high-transmission-efficiency St. Maries strain formed multiple colonies positioned close to host cell granules and containing densely packed bacteria with the bacterial cell morphology associated with the replicative state (11). In contrast, the Israel vaccine strain formed predominately single colonies containing relatively fewer organisms exhibiting the replicative-state morphology. While these observations are, at present, limited to morphology, they support an expanded investigation of strain-specific differences in metabolic and replicative pathways in addition to surface proteins. Furthermore, identifying pathways that lead to salivary secretion may uncover key vector-pathogen interactions underlying transmission phenotypes, with potential for blocking transmission.

Whether the Israel vaccine strain, which is presently classified as *Anaplasma marginale* subsp. *centrale*, is representative of currently circulating low-transmission-efficiency *A. marginale* strains is unknown. However, characterization of multiple *A. marginale* strains has revealed a broad range of transmission phenotypes, including that of the vaccine strain (3, 5, 12, 24, 25). Expanding the investigation to additional wild-type strains is a clear next step to better understanding the basis for strain-specific variations in transmission efficiency and the resulting patterns of strain predominance in the mammalian reservoir host populations.

ACKNOWLEDGMENTS

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