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Protective immunity induced by immunization with a live, cultured *Anaplasma marginale* strain

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Abstract

Despite significant economic losses resulting from infection with *Anaplasma marginale*, a tick-transmitted rickettsial pathogen of cattle, available vaccines provide, at best, only partial protection against clinical disease. The green-fluorescent protein expressing mutant of the *A. marginale* St. Maries strain is a live, marked vaccine candidate (AmStM-GFP[1]). To test whether AmStM-GFP is safe and provides clinical protection, a group of calves was vaccinated, and clinical parameters, including percent parasitized erythrocytes (PPE), packed cell volume (PCV) and days required to reach peak bacteremia, were measured following inoculation and following tick challenge with wild type St Maries strain (AmStM). These clinical parameters were compared to those obtained during infection with the *A. marginale* subsp. *centrale* vaccine strain (*A. centrale*) or wild type AmStM. AmStM-GFP resulted in similar clinical parameters to *A. centrale*, but had a lower maximum PPE, smaller drop in PCV and took longer to reach peak bacteremia than wild type AmStM. AmStM-GFP provided clinical protection, yielding a stable PCV and low bacteremia following challenge, whereas *A. centrale* only afforded partial clinical protection.

Keywords

bovine; tick-borne disease

1. Introduction

Anaplasma marginale is a tick-transmitted rickettsial pathogen of cattle resulting in decreased production due to weight loss, abortion, lower milk yields and death in up to 36% of clinical cases [1]. Despite far-reaching economic impacts there is no vaccine universally

[1]Abbreviations: AmStM: *Anaplasma marginale* St. Maries strain; AmStM-GFP: GFP-transformed AmStM ; OM: Outer membrane

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accepted as safe and efficacious. Various vaccine strategies based on the immunogenic outer membrane proteins of *A. marginale sensu stricto* strains have been examined. Blood-derived whole outer membrane (OM) preparations and cross-linked surface proteins provide the best protection from high level bacteremia and anemia, but may not be practical for large scale production [2-4]. Recombinant proteins, DNA vaccines and killed preparations of *A. marginale*, including inactivated cell-culture derived organisms, have failed to recapitulate the protection seen with OM based vaccines [5-10]. Vaccine induced protection is complex and requires more than antibodies to immunodominant proteins, as studies have repeatedly demonstrated specific seroconversion in the face of failure of clinical protection [5, 10, 11]. Advantages offered by a live vaccine include a full complement of surface antigens in their native conformations, and presentation of new surface protein variants over time.

The *Anaplasma marginale* ssp. *centrale* (*A. centrale*) strain has been used for over a century as a live vaccine against anaplasmosis, and is now widely utilized in Australia, Israel, South Africa and several South American countries to decrease clinical signs associated with exposure to field strains of *A. marginale*. Because the *A. centrale* vaccine is blood-based, it is not approved in the United States or European Union due to the inherent risk of transmission of known or emerging blood-borne pathogens along with the vaccine, as previously demonstrated in a batch of vaccine contaminated with bovine leucosis virus [12]. *A. centrale* protects vaccinates from severe clinical disease upon challenge with field strains of *A. marginale*, with animals generally showing mild signs of anaplasmosis post-vaccination and post-challenge [13, 14]. However, variability in clinical manifestations of anaplasmosis upon infection with the vaccine strain and protection from clinical signs upon challenge with field strains is well documented. Studies in Australia, South Africa, Kenya and Argentina generally demonstrated mild clinical signs post-vaccination and protection against severe disease upon challenge with *A. marginale*, whereas studies in Zimbabwe, Paraguay and Argentina have shown that the same *A. centrale* strain provides little to no protection [15-19]. Potential explanations for variable efficacy include dissimilar endemic strains by country and variation in the challenge dose among studies.

Cross-protection provided by *A. centrale* against challenge with field strains of *A. marginale* is attributed to conserved epitopes [20-22], however there is a much lower degree of conservation between the deduced amino acid sequences of surface proteins of *A. centrale* and sequenced *A. marginale* strains than between any two *A. marginale* strains examined to date. The greater divergence between *A. centrale* and *A. marginale* field strains has been demonstrated in molecular studies: a multi-strain sequencing approach to identify conserved vaccine candidates identified 19 expressed genes with >90% identity among 10 U.S. strains of *A. marginale*. While these sequences all had homologs in *A. centrale*, they were conserved to a much lesser degree, typically between 60-80% [23, 24]. Additional sequence comparisons revealed more divergence among surface proteins between *A. centrale* and *A. marginale* than when comparing just between *A. marginale* strains: 72.4% versus 95.1% average identity [22]. In contrast, housekeeping proteins had higher identities: 97.3% identity when comparing between *A. centrale* and *A. marginale* and 99.7% identity among *A. marginale* strains [22]. These data suggest that better protection may be afforded by a vaccine strain with greater identity to field strains of *A. marginale*.

Here we examine whether a transformed and cell culture-derived *A. marginale* St. Maries strain, more closely related to North American field strains of *A. marginale*, is an alternative approach for safe and effective vaccination. The green fluorescent protein (GFP)-expressing mutant of *A. marginale* St. Maries strain (AmStM-GFP) was created by transposon mediated insertion of a 4.5kb construct containing antibiotic resistance genes for selection and Turbo GFP as a marker, and grows more slowly than the parent strain in culture [25, 26]. The stability of the insert has been demonstrated through a complete *in vivo* transmission cycle

[27]. Two advantages of AmStM-GFP as a vaccine compared to *A. centrale* are its potential to provide better protection due to greater similarity to field strains, and elimination of the risk of delivering emerging pathogens as it is maintained in defined medium in cell culture. In this study we investigate AmStM-GFP as a live, cell culture-based vaccine candidate, and test the hypothesis that infection with AmStM-GFP causes only mild clinical signs and provides clinical protection to vaccinated calves upon challenge with a homologous field strain.

2. Methods

2.1 Cattle inoculation

AmStM-GFP was maintained in ISE6 cells cultured at 34°C as previously described [25, 28, 29]. When passage 27 of AmStM-GFP infected greater than 80% of ISE6 cells in a T75 cell culture flask, as determined by examination of Giemsa-stained cytospin preparations, all cells were re-suspended in 25 ml of media. Three ml aliquots of fresh, intact, unpurified cell culture suspension, each containing 10^9 organisms, were injected intravenously into the jugular vein of each of five male, age-matched, seronegative Holstein calves: 35277, 35340, 35349, 35352, and 35369.

Unpublished clinical data from animal experiments in which naïve calves were infected with either *A. centrale* or the St. Maries strain of *A. marginale* were used in comparisons with AmStM-GFP inoculated calves described above. Five naïve calves were injected with *A. centrale*-infected stabilate, with inoculums containing 10^8 organisms (6171, 6175, 6187, and 6188) or 10^{10} organisms (1302) [30]. *A. centrale* stabilates were prepared from packed erythrocytes, previously washed 3 times in PBS, resuspended in an equal volume of stabilate buffer (1X PBS and 31.2% DMSO), and then plunged into liquid nitrogen. At the time of intravenous injection, 2mL of stabilate were thawed and mixed with 10mL of Hank's balanced salt solution. Fourteen naïve calves (951, 956, 988, 995, 1024, 1067 , 1075, 1076, 1200, 1247, 1280, 31794, 31919, 31993) were infected with AmStM by a 7 day tick-transmission using *Dermacentor andersoni* from the Reynolds Creek stock [31].

All animals were determined to be negative for antibodies to *A. marginale* by competitive ELISA (VMRD, Pullman, WA) prior to experimental infection [32]. Sera from vaccinated and control animals were tested by cELISA to confirm seroconversion after peak bacteremia.

2.2 AmStM challenge

A naïve calf (36676) was inoculated with AmStM stabilate, and infection was established as evidenced by positive Giemsa-stained blood smears and seroconversion. When animal 36676 was in the persistent phase of infection, approximately 460 ticks were applied for a seven day acquisition feed. Ticks were held at 26°C for seven days to allow for clearance of the blood meal from the mouthparts, then 51 ticks were placed on each of four AmStM-GFP infected calves seven months post-inoculation (35277, 35340, 35349, 35352; AmStM-GFP inoculated calf 35369 was removed from the experiment prior to challenge for unrelated health reasons.) and five additional naïve control calves (35294, 35338, 35356, 35370, 35371) for a seven day transmission feed. Following transmission, cohorts of 10 ticks per calf were confirmed positive for AmStM with levels ranging from 10^3 to 10^7 organisms per salivary gland pair.

Calf 1302 was challenged intravenously with 10^9 AmStM blood stabilate-derived organisms thirteen months following inoculation with *A. centrale*.

2.3 Animal monitoring

Following inoculation and challenge, blood samples were collected throughout the period of detectable bacteremia. Blood samples were analyzed by microscopic examination of Giemsa-stained blood smears to determine the level of bacteremia expressed as percent parasitized erythrocytes (PPE) and capillary tube centrifugation to determine packed cell volume (PCV) as a measure of anemia.

Animal experiments were approved by the Institutional Animal Care and Use Committee at Washington State University, USA, in accordance with institutional guidelines based on the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.4 Quantitative PCR

To determine the dose of AmStM-GFP in the inoculum and infection levels in the ticks used for transmission, genomic DNA was extracted from cell culture suspension and tick salivary glands post-transmission using the Puregene DNA Purification cell kit (Qiagen). Quantitative real time PCR of *msp5*, a conserved single-copy gene, was performed with SybrGreen (Invitrogen), and used to determine the number of organisms in each sample. For quantitative amplification, forward 5'-ATA CCT GCC TTT CCC ATT GAT GAG GTA CAT-3' and reverse 5'-AGG CGA AGA AGC AGA CAT AAA GAG CGT-3' primers were used. Standard curves were constructed by amplification of a serial dilution of *msp5* cloned into PCR4-Topo (Invitrogen), and amplified simultaneously with genomic DNA samples. Amplification consisted of an initial 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of melting at 95°C for 15 s, and annealing and extension at 60°C for 1 min, and a final extension at 72°C for 7 min.

2.5 Southern analysis

To detect the presence of AmStM-GFP in vaccinated animals during persistent infection (7 months post inoculation) when the organism is undetectable in blood smears, nested PCR followed by Southern blot targeting *gfp* was performed. Reaction A of the nested PCR was performed with the following primers: GFP F (ATG GAG ATC GAG TGC CGC A) and GFP R (CGG TGT TGC TGT GAT CCT CCT). GFP F2 (ATG ACC AAC AAG ATG AAG AGC ACC A) and GFP R2 (CCG TCC TCG TAC TTC TCG) were used for reaction B of nested PCR and for production of the digoxigenin-labeled probe using the PCR DIG probe synthesis kit (Roche). Amplification for reaction A consisted of thirty five cycles of denaturing at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 30 s, followed by a 7 min extension at 72°C and holding at 4°C. Reaction B differed with a lower annealing temperature of 58.5°C and reduced extension time of 15 s. The Southern blot was performed according to the DIG application manual (Roche).

2.6 Statistics

For days to peak and minimum PCV, mean differences were compared between groups using ANOVA for overall differences between the groups and pairwise comparisons controlling for multiple comparisons using Tukey's test. Because maximum PPE was not normally distributed, median values were compared using the Kruskal-Wallis procedure controlling for multiple comparisons using the Bonferroni-Dunn procedure in WinPepi software [33].

3. Results and Discussion

To determine safety of AmStM-GFP as a vaccine, clinical parameters were compared between animals inoculated with AmStM-GFP versus AmStM or *A. centrale*. When comparing animals inoculated with cultured AmStM-GFP with those inoculated with non-

cultured AmStM, all measured clinical parameters were significantly different. Infection with AmStM-GFP resulted in a lower peak PPE, a smaller drop in PCV, and took longer to reach peak PPE as compared to AmStM (Fig. 1A and 1C). The bacteremia levels measured as PPE were higher in AmStM infected animals than in AmStM-GFP infected animals with non-overlapping ranges of 3.1 to 14.8% and 0.52 to 1.7%, respectively ($p < 0.01$) (Fig. 2A). Anemia as measured by PCV was less severe in AmStM-GFP inoculated animals than in AmStM inoculated animals ($p < 0.01$) (Fig. 2B). The time required to reach peak infection was longer for AmStM-GFP than AmStM with non-overlapping ranges of 41-50 days and 22-37 days, respectively ($p < 0.01$) (Fig. 2C). Our data is consistent with previously reported data that AmStM-GFP grows more slowly than the parent strain in culture, and takes longer to reach peak bacteremia in the bovine host [25, 27]. At no point in the 10 month experiment did any animals experience a reversion to virulence of AmStM-GFP.

When comparing between naïve animals needle inoculated with AmStM-GFP or *A.centrale* stabilate, only one of three clinical parameters was significantly different. AmStM-GFP infected cattle took longer to achieve peak bacteremia than those infected with *A. centrale* (41-50 days vs. 34-46 days) ($p < 0.01$) (Fig. 2C). The maximum PPEs and minimum PCVs between groups were not significantly different (Fig. 2A and 2B), and the infection profiles were similar (Fig. 1A and 1B). The post-vaccination infection profiles indicate that AmStM-GFP results in similar levels of bacteremia and anemia as the *A. centrale* vaccine strain.

Following tick challenge of four AmStM-GFP inoculated animals with wild type AmStM, PCV and PPE were monitored for evidence of transmission to assess protection. PCV fluctuated within the normal range from 26 to 36 throughout 90 days of monitoring (Fig. 3A). Six individual blood smears, representing all four challenged calves at various time points from day 22 to day 52 post-challenge, were positive with calculated PPEs ranging from 0.006% to 0.03%. These positive blood smears were preceded and followed by negative smears on adjacent days and did not correspond to a decrease in PCV. These results are contrasted with those obtained after vaccination with *A. centrale* both in this study (Fig. 3C) and in previously published studies [13, 30], where a characteristic peak of bacteremia associated with a drop in PCV is seen after challenge with *A. marginale*. The *A. centrale* vaccinated animal had measurable bacteremia and associated mild anemia following challenge with AmStM, in contrast to the near absence of microscopically detectable bacteremia observed in the AmStM-GFP vaccinated group (Fig. 3C). That *A. centrale* vaccinated animals become infected with the challenge strain, but are protected from high infection levels and severe anemia has been shown repeatedly [13, 18]. The five control calves were successfully infected by tick challenge as evidenced by seroconversion and development of the expected infection profiles (Fig. 3B). The presence of the vaccine strain was confirmed by molecular methods in all four calves seven months post-vaccination, despite the absence of detectable parasitemia by Giemsa-stained blood smears (Fig. 4).

4. Conclusions

This study describes a live, culture-based vaccine for anaplasmosis using a marked strain of *A. marginale*. Several features of this potential vaccine are noteworthy: 1) Cell culture-based vaccines eliminate the risk of pathogen transmission; 2) Transformation of AmStM has produced a stable, marked, slower growing strain that can be distinguished from field strains (AmStM-GFP was detectable in all four vaccinated calves with negative blood smears seven months post-infection) [25, 27]; 3) Because of the persistent nature of *A. marginale* infection, only a single dose of live AmStM-GFP is required for protection [27]; 4) The findings of this study indicate that AmStM-GFP provides protection against disease following homologous challenge. Further trials are warranted to determine if protection is extended to heterologous challenge. A drawback of this potential vaccine is that it carries an

antibiotic resistance marker, but this could be replaced by an inconsequential marker in future trials. These initial studies are a proof of concept for the basis of future development for this type of vaccine.

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Highlights

- AmStM-GFP is a marked, culture derived, live vaccine candidate for anaplasmosis.
- AmStM-GFP and *A. centrale* result in similar levels of bacteremia and anemia.
- AmStM-GFP provides protection against disease following homologous challenge.

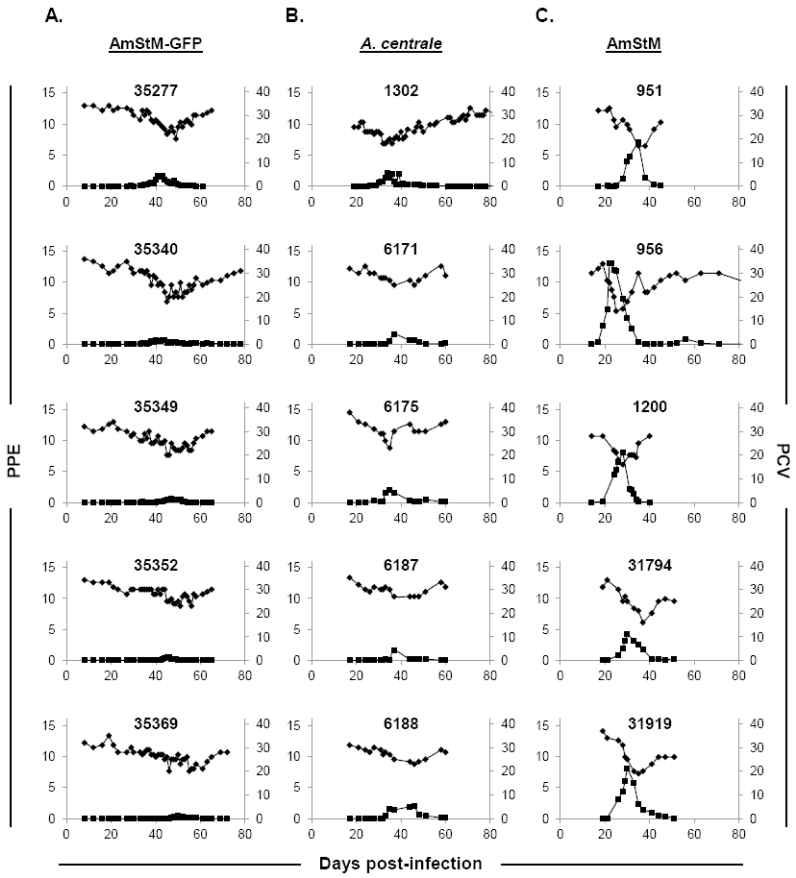

Figure 1.

PPEs and PCVs of AmStM-GFP needle-inoculated (*A*) (n=5), *A. centrale* needle-inoculated (*B*) (n=5), and AmStM tick transmitted calves (*C*) (n=14; 5 represented) during acute anaplasmosis. Animal identification numbers are indicated on each panel. Bacteremia (*left axis*, ■) is reported as percent parasitized erythrocytes as determined by microscopic evaluation of Giemsa-stained blood smears. PCV (*right axis*, ◆) was used to evaluate anemia during infection. The x-axis indicates days post-infection where day 0 is the day of needle inoculation (*A*, *B*) or the day of tick application (*C*). Axes values have been standardized to allow comparison between graphs.

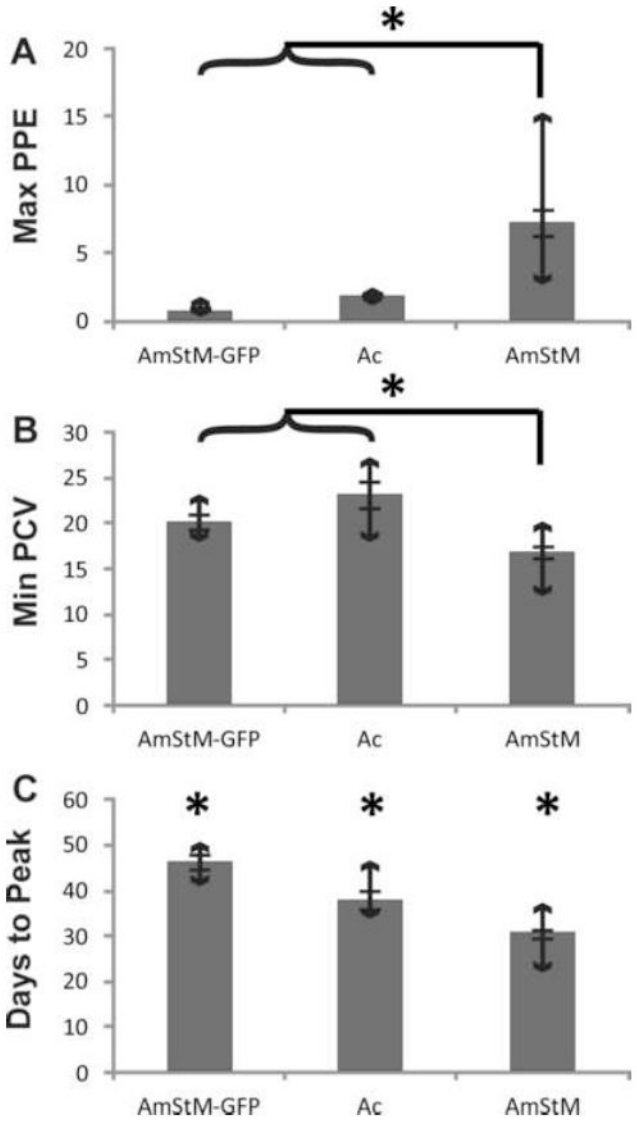

Figure 2.

Clinical parameters of groups of naïve calves needle inoculated with AmStM-GFP (n=5), *A. centrale* (Ac) (n=5) or infected by tick transmission of AmStM (n=14). The group mean is represented by a gray bar, with error bars depicting the standard error. Bars with arrows indicate the range of values for each group. Statistical significance is indicated by an asterisk (*).

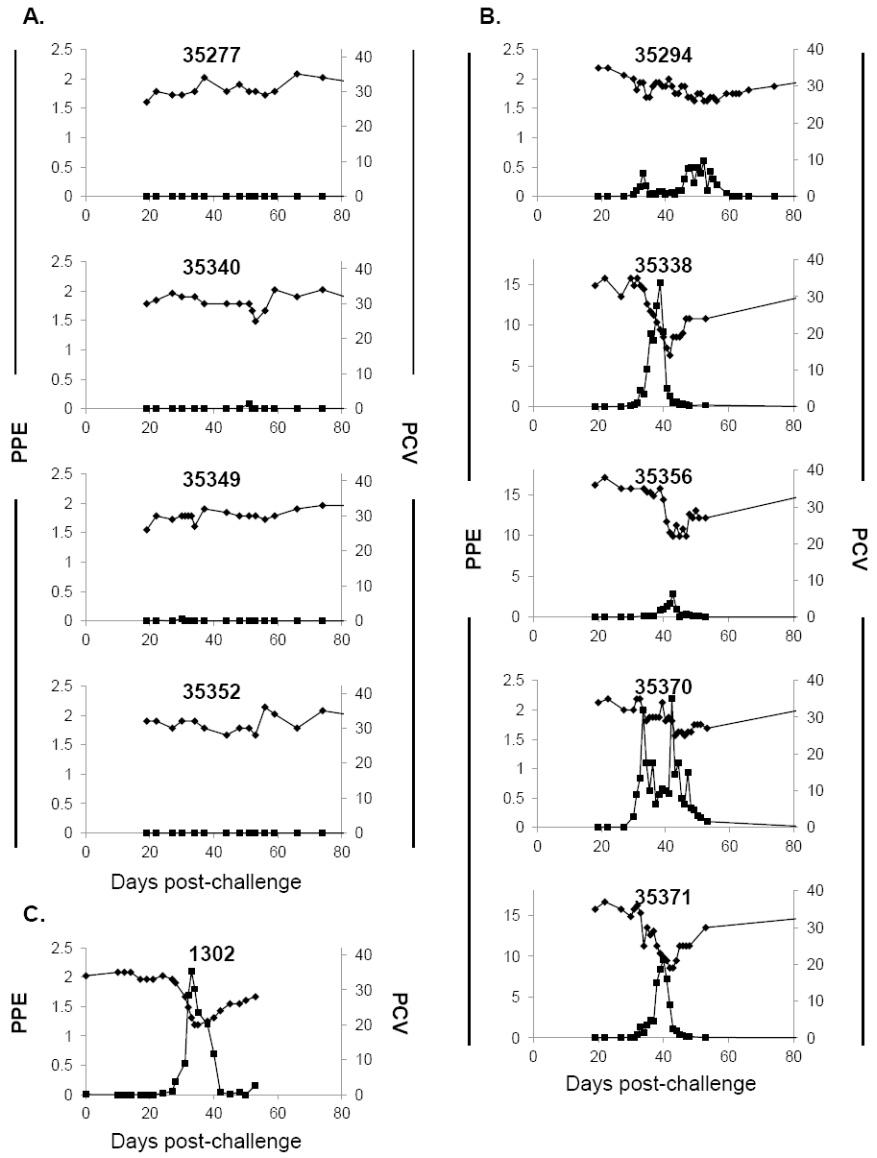

Figure 3.

Clinical parameters following challenge with AmStM. (*A*) Calves inoculated with AmStM-GFP (n=4), (*B*) naïve calves (n=5) and (*C*) *A. centrale* inoculated calf (n=1). Animal identification numbers are indicated on each panel. The x-axis indicates days post-challenge where day 0 is the day of tick application (*A*, *B*) or needle challenge (*C*). Bacteremia (*left axis*, ■) is reported as percent parasitized erythrocytes as determined by microscopic evaluation of Giemsa-stained blood smears. PCV (*right axis*, ◆) was used to evaluate anemia during infection. Axes values have been standardized to allow comparison between graphs, with the exception of the left Y-axis for 35338, 35356 and 35371 in *B*, which were altered to accommodate higher PPE values.

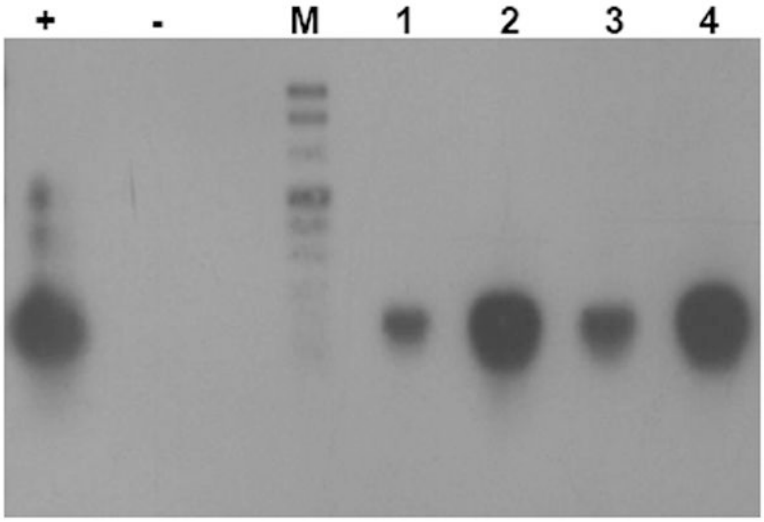

Figure 4.
Southern analysis of *gfp* positive animals during persistent infection. Southern blot following nested PCR targeting *gfp* in genomic DNA extracted from calf blood seven months post-vaccination: (+) positive plasmid control, (-) negative water control, (M) DNA Molecular Weight Marker VIII, DIG-labeled (Roche), (1) calf #35277, (2) calf #35340, (3) calf #35349, (4) calf #35352.