Imidocarb Dipropionate Clears Persistent Babesia caballi Infection with Elimination of Transmission Potential

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Antimicrobial treatment of persistent infection to eliminate transmission risk represents a specific challenge requiring compelling evidence of complete pathogen clearance. The limited repertoire of antimicrobial agents targeted at protozoal parasites magnifies this challenge. Using Babesia caballi as both a model and a specific apicomplexan pathogen for which evidence of the elimination of transmission risk is required for international animal movement, we tested whether a high-dose regimen of imidocarb dipropionate cleared infection from persistently infected asymptomatic horses and/or eliminated transmission risk. Clearance with elimination of transmission risk was supported by the following four specific lines of evidence: (i) inability to detect parasites by quantitative PCR and nested PCR amplification, (ii) conversion from seropositive to seronegative status, (iii) inability to transmit infection by direct inoculation of blood into susceptible recipient horses, and (iv) inability to transmit infection by ticks acquisition fed on the treated horses and subsequently transmission fed on susceptible horses. In contrast, untreated horses remained infected and capable of transmitting B. caballi using the same criteria. These findings establish that imidocarb dipropionate treatment clears B. caballi infection with confirmation of lack of transmission risk either by direct blood transfer or a high tick burden. Importantly, the treated horses revert to seronegative status according to the international standard for serologic testing and would be permitted to move between countries where the pathogen is endemic and countries that are free of the pathogen.

Antimicrobial therapy is primarily directed to reducing pathogen load below levels associated with disease, and treatment efficacy is most commonly evaluated by improvement in clinical signs (23, 27). Asymptomatic persistent infections represent an important subset of infections and present specific challenges for antimicrobial therapy (21, 24). The goal of therapy in persistent infections is clearance of the pathogen to prevent future relapse to clinical disease and/or transmission to additional susceptible hosts. Thus, achieving and confirming pathogen clearance become paramount in the treatment of persistent infections.

The taxonomic range of pathogens that establish asymptomatic persistent infection is extremely broad, from RNA viruses to eukaryotic parasites (8, 15, 26). Among the latter, apicomplexan parasites in the genera Babesia, Plasmodium, and Theileria illustrate both the difficulty of effecting clearance with a limited repertoire of antimicrobial drugs and confirming that clearance and the elimination of subsequent transmission risk have been achieved (10, 21, 25, 29). These pathogens may persist in immunocompetent hosts at levels below the limits of routine microscopic detection and without overt signs of disease and yet serve as efficient reservoirs for arthropod vectorborne transmission (10, 19, 26, 28). Babesia caballi exemplifies this pattern: horses that recover from acute disease, when parasitemia levels exceed $10^6$ parasites per ml of blood, progress to an asymptomatic phase with parasitemia below $10^3$ parasites per ml of blood (18, 26). Acute B. caballi infection is characterized by high fever (>40°C), anemia, anorexia, malaise, tachypnea, and dyspnea (9). Following the acute phase, horses remain persistently infected and serve as reservoirs for transmission by tick vectors (26). Areas of endemicity for B. caballi include parts of Africa, the Middle East, Asia, Central and South America, the Caribbean, and Europe (9). While this hemoprotozoan parasite is widespread in tropical and subtropical regions, infecting horses, mules, donkeys, and zebras, many temperate-region countries are free of B. caballi infection and prohibit entry of infected horses (14). Consequently, the importation of horses into B. caballi-free countries or regions requires clearance of infection from persistently infected asymptomatic horses and confirmation of infection-free status. This requirement has a significant impact on the international movement of horses highly valued for either breeding or competition (5, 14). In this study, we tested whether imidocarb dipropionate eliminated B. caballi from persistently infected horses and, consequently, the risk of transmission by either direct blood transfer or tick vectors (the natural route of transmission). Furthermore, we tested if imidocarb dipropionate treatment resulted in reversion to seronegative status accord-
ing to the international standard for importation of horses into infection-free countries.

MATERIALS AND METHODS

Animals, pathogen, and tick vector. The horses used in this study were determined to be free of B. caballi infection by rhoptry-associated protein 1 (RAP-1) competitive enzyme-linked immunosorbent assay (C-ELISA; VMRD) and rap-1 nested PCR as previously described (20, 26). The Puerto Rico strain of B. caballi was used for all infections (26). Larval offspring of Dermacentor nitens, initially collected in Puerto Rico, were fed on calves (nonsusceptible hosts for B. caballi infection) through three consecutive generations to establish a B. caballi-free colony as previously described (26).

Infection with B. caballi. Four naïve horses (Ho-133, Ho-145, Ho-146, and Ho-147) were infected by inoculation with 10^5.2 parasites and monitored daily for clinical signs. Infection was detected and quantified using both microscopic examination of Giemsa-stained blood smears and by PCR. For PCR, genomic DNA was isolated from 100 μl of blood by using a Puregene DNA purification system and resuspended in 50 μl of Tris-EDTA buffer (Gentra Systems). Infection with B. caballi was confirmed by rap-1 nested PCR. The absence of PCR inhibition was determined by detection of equine β-actin as previously described (26). For PCR quantification, a standard curve was developed using dilutions of known copy numbers of a plasmid containing the rap-1 gene. To construct the plasmid, genomic DNA was extracted from the B. caballi Puerto Rico strain. Full-length gene amplification was performed using the following primer set: forward, 5'-TGT GTC-3', and reverse, 5'-CCA AAG ATT CCA CAG-3'. Amplification used cycles of 95°C for 5 min; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min; final extension at 72°C for 7 min; and holding at 4°C. The amplified rap-1 product was cloned into the pCR4-TOPO vector, and competent TOP10 Escherichia coli cells were transformed (Invitrogen). Plasmid DNA was isolated (Promega), and the presence of inserts confirmed by EcoRI restriction enzyme digestion. Then, the inserts were sequenced in both directions using a BigDye Kit and an ABI Prism automated sequencer (Applied Biosystems). Sequencer (Gene Codes) was used to assemble and edit the rap-1 plasmid sequence (GenBank accession number EU669865). For the quantitative PCR, a TaqMan assay was performed utilizing Universal Probe Library number 37 (Roche Applied Science) and a primer set (forward, 5'-ACA ATG AGG TGT TCT GCG AGT TC-3', and reverse, 5'-TAG TCA CGT CGC CCA CAG AGT-3') under the following conditions: 95°C for 10 min; 50 cycles of 95°C for 30 s, 60°C for 20 s, and 72°C for 20 s; final extension at 72°C for 1 min; and holding at 4°C. In repeated runs, the r² value of the standard curve ranged from 0.95 to 1.00.

The quantitative PCR results were compared with those of microscopic examination of Giemsa-stained blood smears to test for concordance between assays. There was ≤0.5 log₁₀ difference between the numbers of B. caballi parasites detected by quantitative PCR and the numbers found by microscopic examination of blood smears (Fig. 1).

Antimicrobial treatment. Following the resolution of the acute phase of infection, during which parasitemia levels exceeded 10^6 organisms per ml of blood, all infected horses progressed to persistent infection with parasitemia levels of <10^5 organisms per ml of blood (Fig. 2). During the persistent phase of infec-
tion, 70 days postinfection, horses Ho-145 and Ho-147 were treated with imidocarb dipropionate (Imizol; Schering Plough Animal Health) via intramuscular injection at a dose of 4.0 mg per kg of body weight four times at 72-h intervals. Persistently infected horses Ho-133 and Ho-146, initially infected at the same time, were left untreated and used as negative treatment controls.

Detection of specific antibodies against *B. caballi*. Serum samples from untreated horses and horses treated with imidocarb dipropionate were analyzed using RAP-1 C-ELISA, the complement fixation test (CFT), and immunoblot assay to determine whether antimicrobial treatment resulted in conversion from seropositive to seronegative status (3, 13, 20). For C-ELISA, undiluted serum samples from treated and untreated horses were tested in duplicate using a recombinant RAP-1-coated plate (VMRD) as previously described (20). For CFT, serum samples from treated and untreated horses were diluted 1:5 in veronal-buffered saline and tested according to methods previously described (13). For the immunoblot assay, approximately 100 ng of *B. caballi* Puerto Rico strain lysate was loaded per lane and proteins were separated by electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 10% skim milk in phosphate-buffered saline with 0.2% Tween 20 (PBST). Serum samples were diluted 1:100 in PBST and incubated for 30 min at room temperature. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-horse (Kirkegaard & Perry Laboratories) immunoglobulin (diluted 1:5,000 in PBST). Following washing, bound antibodies were visualized by using a chemiluminescence ECL Plus kit (General Electric).

Transmission by direct blood inoculation. To determine whether the antimicrobial therapy eliminated circulating *B. caballi*, peripheral blood from treated and untreated horses was individually inoculated into naive horses. Three months after treatment, 100 ml of blood collected in EDTA from each of the horses was inoculated into the jugular vein of a naive horse. The pairs of horses were as follows (blood donor animal/recipient animal): Ho-145/Ho-126, Ho-147/Ho-127, Ho-133/Ho-121, and Ho-146/Ho-122. Recipient horses were monitored daily for clinical signs of infection. Blood and serum samples were tested for the presence of *B. caballi* and seroconversion using, respectively, nested PCR and C-ELISA (26).

Transmission by tick feeding. To determine whether the antimicrobial therapy cleared *B. caballi* and removed the risk of tick-borne transmission, transmission-competent ticks were acquisition fed on treated or untreated horses and the next generation fed on naive horses. Five months after antimicrobial treatment, approximately 20,000 uninfected *D. nitens* larvae were acquisition fed on treated horses (Ho-145 and Ho-147) and untreated controls (Ho-133 and Ho-146). The ticks were allowed to acquire feeding through one complete generation (larvae, nymphs, and adults) on each animal. Engorged *D. nitens* females were collected from each horse and incubated for ovoposition as previously described (26). Approximately 20,000 larvae of the subsequent generation were allowed to transmission feed for a complete generation (larvae, nymphs, and adults) on each of five naive horses. The pairs of horses used for the acquisition and transmission feeding, respectively, were as follows: Ho-133/Ho-150, Ho-145/Ho-154, Ho-146/ Ho-155, and Ho-147/Ho-153. *D. nitens* females were collected from each horse and incubated for ovoposition as previously described (26).

Additional transmission of naive horses. Larval offspring from female *D. nitens* that had been acquisition fed through one complete tick generation on imidocarb dipropionate-treated horses failed to transmit *B. caballi* to naive horses (Table 1). In contrast, larval offspring from females identically acquisition fed on untreated horses successfully transmitted *B. caballi* to naive recipient animals, with detection of *B. caballi* infection within 12 days of tick feeding (Table 1) and progression to a peak parasitemia level of $10^6$ parasites per ml of blood. Larval offspring from females identically fed on the negative control horse Ho-105 did not transmit *B. caballi* to a recipient animal.

**FIG. 3.** Clearance of *Babesia caballi* infection following antimicrobial treatment. (a) Nested PCR targeting *B. caballi* rap-1. (b) Nested PCR targeting equine β-actin. Horses 133 and 146 were untreated; horses 145 and 147 were treated with imidocarb dipropionate. The leftmost lanes contain molecular size ladders. inoc, inoculation.
DISCUSSION

Antimicrobial treatment of *Babesia caballi*-infected horses with the high-dose regimen of imidocarb dipropionate eliminated the parasite and, consequently, the transmission risk. This conclusion is supported by four specific lines of evidence: (i) inability to detect parasites by quantitative PCR and nested PCR amplification, (ii) conversion from seropositive to seronegative status, (iii) inability to transmit infection by direct inoculation of 100 ml of blood into susceptible recipient horses, and (iv) inability to transmit infection by ticks acquisition fed on the treated horses and then subsequently transmission fed on susceptible horses. In contrast, untreated horses remained infected and capable of transmitting *B. caballi* using the same criteria. Of these four, the last two are the most compelling and most relevant indicators of clearance. *B. caballi* parasitemia detected during long-term persistence in the infected horses fluctuated between $10^2$ and $10^5$ parasites per ml of blood. Thus, direct inoculation of 100 ml of blood without treatment would transmit a minimum of $10^4$ parasites. As the minimal infectious dose for babesial parasites is $\leq 100$ organisms (16), the inability of treated horses to transmit infection by direct inoculation suggests a minimal reduction of $\approx 99\%$ and a maximal reduction of $\approx 99.999\%$ in numbers of circulating parasites and indicates that direct transfer of minute quantities of blood by biting flies or other fomites would carry no risk of transmission. The results in the current study were consistent with those of previous studies in which direct inoculation of blood from imidocarb dipropionate-treated horses failed to infect susceptible animals (7, 12).

Alone, the inability to transmit *B. caballi* by direct inoculation is not evidence of clearance as very low numbers of parasites could, conceivably, persist either in the circulation or sequestered in tissue capillaries following antimicrobial treatment. Similar to several other species in the genera *Babesia* and *Plasmodium*, *B. caballi* may sequester in small capillaries and then later reemerge into the circulation (2, 4). Tick feeding can convey the disease as well. The results of this study are consistent with those of previous studies in which direct inoculation of blood from imidocarb dipropionate-treated horses failed to infect susceptible animals (7, 12).

![FIG. 4. Detection of specific antibody against *Babesia caballi* following antimicrobial treatment. I. blot, immunoblot.](image)

| TABLE 1. Assessment of transmission of *Babesia caballi* via direct blood transfer and tick feeding following antimicrobial treatment |
|-----------------------------|-----------------------------|-----------------------------|
| **Donor horse** | **Recipient horse** | **Transmission model** | **Pretransmission** | **Posttransmission (no. of days)** |
| | | | **Nested PCR** | **C-ELISA** | **Nested PCR** | **C-ELISA** |
| Ho-133$^a$ | Ho-121 | Intravenous | – | – | + (8) | + (8) |
| Ho-146$^a$ | Ho-122 | Intravenous | – | – | + (8) | + (8) |
| Ho-145$^a$ | Ho-126 | Intravenous | – | – | (>100) | (>100) |
| Ho-147$^a$ | Ho-127 | Intravenous | – | – | (>100) | (>100) |
| Ho-133$^b$ | Ho-150 | Tick borne | – | – | + (12) | + (12) |
| Ho-146$^b$ | Ho-155 | Tick borne | – | – | (>100) | (>100) |
| Ho-145$^b$ | Ho-154 | Tick borne | – | – | (>100) | (>100) |
| Ho-147$^b$ | Ho-153 | Tick borne | – | – | (>100) | (>100) |
| Ho-105$^c$ | Ho-137 | Tick borne | – | – | (>100) | (>100) |

$^a$ Untreated horse.

$^b$ Horse treated with imidocarb dipropionate.

$^c$ Uninfected (negative control) horse.
has been shown to transmit pathogens, including those that sequester in small capillaries (19), from persistently infected mammalian reservoir hosts with parasite levels below the detection limits of culture, PCR amplification, or direct inoculation (11, 17). Natural vectors, such as Dermacentor nitens for B. caballi, efficiently acquire the parasite from small capillaries during the feeding process and then amplify the number of organisms within the tick via replication in the salivary glands prior to transmission (26). The inability to transmit infection using 20,000 D. nitens ticks strongly supports that clearance was effected by the treatment and, most importantly, indicates that tick transmission, even under a very heavy tick burden, does not occur.

Following antimicrobial treatment, B. caballi parasitemia decreased within 5 days to undetectable levels in the peripheral blood as monitored by PCR. The results of pharmacokinetic studies indicate that imidocarb dipropionate is rapidly absorbed, reaches high levels in the plasma, and is broadly distributed at the tissue level, including kidney, liver, and brain (1, 29), organs which may sequester B. caballi parasites. Whether the immediate effect on peripheral parasitemia revealed by the PCR assay indicates that transmission risk, either by direct blood transfer or by tick transmission, is also reduced in the same time frame was not addressed by the study as the transmission experiments were conducted months after the treatment. However, such studies would be critical to establish a quarantine period based on experimental data for prevention of transmission following antimicrobial treatment. In contrast to the data presented here, a recent study using naturally infected horses reported recrudescence parasitemia following imidocarb dipropionate treatment (6). One possible explanation for this discrepancy is that there are strain differences in imidocarb susceptibility, either constitutive or due to resistance induced by prior exposure to subclearing doses. The strain used in the present study had no known prior exposure to imidocarb. However, to date there are no studies that directly address the development of resistance to imidocarb by B. caballi.

The conversion from seropositive to seronegative status using a sensitive C-ELISA is the fourth level of proof for treatment-induced clearance. Even in the event of sequestration of a small number of parasites not detectable in the peripheral blood, parasite antigens would continue to stimulate the existing B-cell response and trigger ongoing antibody secretion (19). The time lag between treatment and conversion to seronegativity using the C-ELISA and immunoblot assay was consistent with the levels of antibody in the individual horses prior to treatment and the half-lives of equine immunoglobulins (30). Importantly, once seronegative, the horses remained uniformly negative using the C-ELISA and immunoblot assay, in agreement with the sensitivity and specificity of the assays. The C-ELISA is an international standard diagnostic assay established by the World Animal Health Organization (OIE) and is currently required for movement of horses into countries free of B. caballi (20). The concordance of the lack of direct or tickborne transmission with the C-ELISA results supports the accuracy of this assay in establishing the infection and transmission risk status of imported horses. This stands in contrast to the previously used CFT, which provides inconsistent results during persistent infection due to a lack of sensitivity (5, 22).

Interestingly, during the course of the persistent phase of infection, B. caballi levels consistently fluctuated in the range from 10^10 to 10^12 parasites/ml of blood. We hypothesize that this cyclic parasitemia reflects either (i) sequential waves of antigenic variants with each cycle controlled by a variant-specific immune response followed by new variant emergence or (ii) sequential waves of sequestration in tissue capillaries followed by reemergence and replication. These hypotheses are not mutually exclusive as parasite antigens expressed on the surface of infected erythrocytes have been shown to mediate both antigenic variation with escape from immune clearance and specific sequestration (2, 4). These events very likely underlie the capacity of this parasite to persist at low levels and for the infected horse to serve as an efficient reservoir host for tick transmission.

Treatment of protozoal infections, directed at either relief of clinical disease or clearance of persistent infection and transmission risk, remains a challenge due to the limited array of chemotherapeutic agents and narrow therapeutic margin. Nonetheless, our data indicate that aggressive imidocarb dipropionate treatment clears B. caballi from persistently infected horses and both meets the regulatory standard for international movement of horses and eliminates transmission risk by tick vectors.

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