The therapeutic efficacy of two antibabesial strategies against Babesia gibsoni

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Various combination strategies for treating Babesia gibsoni have been described. However, relapses after administering some combinations of antibabesial drugs and the presence of drug-resistant B. gibsoni still pose significant challenges to veterinarians. To compare the efficacy of a combination of clindamycin, diminazene, and imidocarb (CDI) to that of a combination of atovaquone and azithromycin (AA) for the treatment of B. gibsoni and to correlate drug efficacy with B. gibsoni mutations, 30 client-owned dogs with natural B. gibsoni infections were collected in the study. 17 dogs were treated with AA, and 13 dogs were treated with CDI combination. Hematological parameters were recorded on the day that the dogs were presented for treatment and during treatment. To detect the parasitic DNA, the B. gibsoni 18S rRNA gene was amplified, and to analyze the mutations, the cytochrome b (CYTb) gene was sequenced. The therapy duration for all of the dogs that recovered was 23.3 ± 7.8 days in the AA group and 41.7 ± 12.4 days in the CDI group. Nine of the 17 dogs in the AA group and 11 of the 13 dogs in the CDI group completely recovered. Seven dogs in the AA group and 2 dogs in the CDI group relapsed after treatment. The M121I mutation in the B. gibsoni CYTb gene was detected in all of the samples that were collected from AA-relapsed and AA-nonremission dogs. The dogs in the CDI group exhibited higher recovery rates and lower relapse rates during treatment for B. gibsoni infection. In addition, the detected M121I mutation was associated with AA treatment. The CDI combination is a promising alternative treatment strategy for B. gibsoni.

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1. Introduction

Canine babesiosis, which is caused by hemoproteozoan organisms of the genus Babesia that include both Babesia conis and Babesia gibsoni, is a life-threatening, tick-borne disease, and the elimination of this pathogen, especially its Asian genotype, has been a perennial challenge for clinical veterinarians (Birkenheuer et al., 2004; Jefferies et al., 2007). Several drugs have been used to treat B. gibsoni infections; however, most studies have revealed that no single drug is sufficient for the treatment of this disease (Matsu et al., 2004; Wulansari et al., 2003a,b; Suzuki et al., 2007; Birkenheuer et al., 1999). Therefore, drug combinations appear to be a better choice for treating B. gibsoni infections. Birkenheuer et al. (2004) found that a combination of atovaquone and azithromycin is an effective therapeutic strategy for suppressing parasitemia in the B. gibsoni Asian genotype; however, B. gibsoni could still be detected by PCR after this treatment (Birkenheuer et al., 2004). Furthermore, relapses frequently occurred after
treatment with the AA combination (Sakuma et al., 2009), and the efficacy of atovaquone is known to be influenced by various mutations, particularly the M121I mutation, in the *B. gibsoni* cytochrome b (CYTb) gene (Matsuu et al., 2006). Doxycycline in combination with diminazene aceturate has also been prescribed to treat *B. gibsoni*; however, a recent study found that persistent parasitemia can still be detected in blood smears after this treatment (Birkenheuer et al., 1999). In another study, a combination of clindamycin, doxycycline, and metronidazole was successfully used to treat the disease without signs of relapse in 3 out of 4 splenectomized dogs that were experimentally infected with *B. gibsoni*; however, one dog showed persistent parasitemia throughout the experiment (Suzuki et al., 2007). According to the literature, no current therapeutic strategy has been found to completely eliminate *B. gibsoni* infections.

The objectives of this study were to establish a clinically effective therapeutic strategy that combined clindamycin, diminazene aceturate, and imidocarb dipropionate (CDI) and to compare the efficacy of this treatment with that of a combination of atovaquone and azithromycin (AA) for the treatment of *B. gibsoni*. We measured and compared PCV, Hb, RBC, platelets, and parasitemia in animals that were treated with either CDI or AA. Moreover, the *B. gibsoni* CYTb gene was sequenced to determine the effects of *B. gibsoni* mutations and to provide a useful indicator for the selection of a therapeutic strategy.

2. Materials and methods

2.1. Study design

This study was a prospective, unmasked clinical trial that placed dogs into two treatment groups; the dogs received either the AA or CDI combination. The dogs were placed into their respective groups based on the wishes of their owners after the costs, probable side effects and therapeutic durations of the two treatments had been explained.

2.2. Criteria for case selection

For inclusion in the study, the dogs were required to have been diagnosed with babesiosis; this diagnosis was confirmed by a microscopic *B. gibsoni* – positive blood smear and the presence of anemia. All the dogs were presented at the National Taiwan University Veterinary Hospital (NTUVH) between June 2006 and October 2009. All the dogs were under continual tick prevention after the therapy began. The presence of concurrent diseases in the animals evaluated in this study included heart disease in 5 dogs, nephrolithiasis in one dog and positive *Ehrlichia canis* antibody (detected by IDEXX SNAP kit) in one dog. In this study, recovery was defined as the presence of both normal clinical signs and hematological examinations, including the absence of parasitemia in a blood-smear examination and/or a negative PCR result. In contrast, relapse was defined as the reappearance of associated clinical signs and hematological abnormalities, including anemia and parasitemia in a blood film and/or a positive PCR result after remission from the initial therapy. Nonremission was defined as persistent parasitemia in microscopic blood-smear examinations or positive PCR tests associated with persistent anemia during treatment.

2.3. Hematological and PCR examinations

Complete blood counts (CBCs), blood-smear examinations by light microscopy, and biochemistry (i.e., albumin, total protein, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, blood urine nitrogen, creatinine, and glucose levels) were analyzed on the presenting day (day 0). The Hb, PCV, RBC, platelets, and parasitemia values were recorded 7, 14, 21, and 28 days after the dogs presented with the disease. Multiplex-nested PCR was performed on 22 of the 30 dogs, and the *B. gibsoni* CYTb gene was sequenced in 16 of these animals.

2.4. Treatment protocols

The included dogs were divided into two groups, according to the treatment they received. The AA group was treated with a combination of atovaquone (Mepron, Glaxo SmithKline, Middlesex, UK; 13.3 mg/kg body weight, PO, q8 h) and azithromycin (Zithromax, Pfizer, Italy; 10 mg/kg body weight, PO, q24 h), whereas the CDI group was treated with a combination of clindamycin phosphate (Tidact, Yung Shin Pharmaceutical Industrial Co. Ltd., Taichung, Taiwan; 30 mg/kg body weight, PO, q12 h), diminazene aceturate (Dimaphen, Phenix Pharmaceutical N.V., Belgium; 3.5 mg/kg body weight, IM, a single dose on the day of presentation), and imidocarb dipropionate (Imizol, Schering-Plough animal health, Germany; 6 mg/kg body weight, SC, a single dose on the day after the diminazene was administered). The point at which therapy ceased was determined using blood examinations (when the PCV reached the median of the reference range for at least one week), and a serial negative blood-smear examination was performed for at least two weeks during the therapy.

2.5. Multiplex-nested PCR amplification of the *B. gibsoni* 18S rRNA gene

The nucleotides of the 18S rRNA gene, which were collected from blood samples that were obtained before and/or after treatment and/or at relapse, were analyzed. In the first round of PCR, 2 μl of the DNA was added to 28 μl of the PCR mixture. The PCR mixture consisted of 3 μl of 10× Taq buffer, 0.5 μl of each primer (5′-CTACCACTCTAAGGAAAGC-3′ and 5′-TGCTTTGCCAGTATGTCGTC-3′), 1 μl dNTPs (2.5 mM), 0.5 μl Taq DNA polymerase (GeneTeks BioScience, Inc., Taipei), and 22.5 μl of 0.1% DEPC-treated water. The first-round PCR amplification was performed under the following conditions: 3 min of preheating at 94°C; 35 cycles of denaturation at 94°C for 20 s; annealing at 63°C for 20 s and extension at 72°C for 35 s; a final extension at 72°C for 5 min. Multiplex-nested PCR was performed on 1 μl of the first PCR product using nested primers (5′-TGTTTCCAGTATGTCGTC-3′ (*Babesia* spp.), 5′-GTGAAATTCTCGCTGCCC-3′ (*B. gibsoni* Asian genotype), and 5′-AGTGCCAATTCGTGTTGG-3′ (*B. canis*). The
second-round PCR amplification was performed under the following conditions: 3 min of preheating at 94 °C; 35 cycles of denaturation at 94 °C for 20 s; annealing at 63 °C for 20 s; extension at 72 °C for 20 s; a final extension at 72 °C for 5 min. The first-round PCR yielded the expected products for B. canis, B. gibsoni, North American genotype, and Asian genotype, which were 490, 520, and 490 bp in length, respectively. The second-round PCR yielded the expected products for the B. canis and B. gibsoni Asian genotype, which were 249 and 268 bp in length, respectively. Positive and negative controls were performed for each step.

2.6. Nested PCR and B. gibsoni CYTb gene sequencing

The B. gibsoni CYTb gene was detected via PCR, as previously described (Sakuma et al., 2009). The nucleotide sequences of the amplified DNA fragments were determined via direct sequencing with an automated DNA sequencer. DNASTAR Lasergene was used to characterize the obtained nucleotide-sequence data.

2.7. Statistical analysis

The data in this study are expressed as mean ± standard deviation (SD), and the hematological data are expressed as medians and ranges. The two groups were tested using chi-square tests for independence. The statistical differences in the numeric variables (Hb, PCV, RBC, platelet counts, and parasitemia) between the AA and CDI groups were assessed using Wilcoxon’s rank-sum tests and standard statistical software. Differences were considered to be statistically significant when their associated p-values were <0.05.

3. Results

3.1. Clinical parameters and hematological examinations

The 30 dogs included 21 males (70%) and 9 females (30%). Their ages ranged from 0.5 to 13 years old (5.1 ± 4.0 years), and their body weights (BWs) ranged from 1.75 to 37 kg (10.88 ± 9.9 kg). Seventeen and 13 dogs were enrolled in the AA and CDI groups, respectively, and there were no differences in ages or BWs of the two groups (p = 0.78 and 0.057, respectively). Table 1 depicts the severity of the anemia in both groups. The hematological characteristics of the dogs in the AA group on day 0 were as follows: Hb, 6.6 g/dL (range, 3.1–12.4 g/dL); PCV, 19.9% (range, 10.8–36.8%); RBC, 2.61 × 10⁹ μL⁻¹ (range, 1.16–5.21 × 10⁹ μL⁻¹); platelets, 49 × 10³ μL⁻¹ (range, 10–196 × 10³ μL⁻¹); parasitemia, 1.08% (range, 0.12–46.15%). The dogs in the CDI group exhibited the following hematological characteristics on day 0: Hb, 7.40 g/dL (range, 3.6–13.8 g/dL); PCV, 22.4% (range, 12.3–35.1%); RBC, 2.95 × 10⁹ μL⁻¹ (range, 1.36–5.51 × 10⁹ μL⁻¹); platelets, 63 × 10⁹ μL⁻¹ (range, 20–170 × 10³ μL⁻¹); parasitemia, 0.60% (range, 0.10–8.33%). There were no statistical differences in these measurements between the two groups on the presenting day (Table 2).

Serial hematological examinations were also performed on days 7, 14, 21, and 28 (Table 2). The Hb, PCV, and RBC counts were significantly different between the two groups on day 7 (p = 0.013, 0.040, and 0.029, respectively). The platelet levels increased from 49 to 370 × 10³ μL⁻¹ in the AA group and from 63 to 179 × 10³ μL⁻¹ in the CDI group on day 7; this difference between the two groups was significant (p = 0.00094). The platelet counts markedly increased in the AA group; however, the observed increases in the Hb, PCV, and RBC values were not significantly different between the two groups (p = 0.072, 0.051, and 0.086, respectively). On days 14, 21, and 28, there were no differences in the Hb, PCV, RBC, and platelet values between the two groups. Moreover, on day 28, the Hb, PCV, and RBC values were higher in the CDI group than in the AA group. Parasitemia dramatically decreased in both groups, and there were no significant differences between the two groups on the presenting day or on subsequent days.

Nine of the 17 dogs in the AA group and 11 of the 13 dogs in the CDI group completely recovered from their illnesses, with no relapses after treatment. The therapy duration for all of the dogs that recovered was 23.3 ± 7.8 days in the AA group and 41.7 ± 12.4 days in the CDI group; this difference in the therapy durations of the two groups was significant (p = 0.0068). Seven of the 17 dogs (cases 3, 4, 8, 10, 11, 13, and 15) in the AA group and 2 of the 13 dogs (cases 25 and 27) in the CDI group relapsed after therapy. In case 25, reduced appetite and hemoglobinuria were present, and the PCV decreased from 42.2% to 39.7%, with both positive blood smear and PCR results on day 56, and a second round of CDI therapy was administered for another 63 days. At the time of this writing, this dog has recovered and shown no signs of relapse. Case 27 relapsed on day 77 with acute babesiosis; its PCV decreased from 44.3 to 25.5%. AA combination treatment was administered for 21 days, and the dog recovered.

3.2. Genotyping of B. gibsoni

During the initial presentation, 8 of the 17 dogs in the AA group (cases 7, 9, 10, 11, 13, 14, 16, and 17) and 8 of the

Table 1
Hematological abnormalities in 30 B. gibsoni–infected dogs at presentation.

<table>
<thead>
<tr>
<th>Item</th>
<th>Group</th>
<th>Numbers of dogs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA (n = 17)</td>
<td>CDI (n = 13)</td>
</tr>
<tr>
<td>Anemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild (25 &lt; PCV &lt; 37%)</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>Moderate (15 &lt; PCV &lt; 25%)</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Severe (PCV &lt; 15%)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Thrombocytopenia (&lt;20,000 μL)</td>
<td>17</td>
<td>13</td>
</tr>
</tbody>
</table>
13 dogs in the CDI group (cases 20, 21, 22, 23, 25, 26, 27, and 29) were all simultaneously diagnosed with the Asian genotype of the *B. gibsoni* infection by mPCR. Six of the 7 relapsed dogs in the AA group (cases 3, 4, 8, 11, 13, and 15) and 2 relapsed dogs in the CDI group (cases 25 and 27) were also confirmed to have the *B. gibsoni* Asian genotype at the time of relapse.

3.3. Sequencing and analysis of the *B. gibsoni* CYTb gene

The isolation and sequencing of the CYTb gene (from nt 93 to 370) were performed on 12 dogs (cases 7, 9, 11, 13, 14, 20, 22, 23, 25, 26, 27, and 29) when they first presented. The M121I mutation in the *B. gibsoni* CYTb gene was detected in case 9, which had been treated with atovaquone therapy five months prior to presentation. A single-nucleotide substitution (from ATA to GTA) was detected at nucleotide 325 in case 26, resulting in an I109V mutation. Twelve blood samples from eight dogs (cases 3, 4, 8, 11, 13, 15, 25, and 27) that had relapsed were sequenced, and the M121I mutation in the *B. gibsoni* CYTb gene was detected in all of the relapsed dogs from the AA group (Table 3); however, no mutation in this region was detected in the relapsed dogs from the CDI group.

4. Discussion

More than 100 cases that were genotyped for *Babesia* in the NTUHV lab between 2005 and 2010 were identified as the *B. gibsoni* Asian genotype (data not shown), suggesting that the *B. gibsoni* Asian genotype is the major pathogen responsible for canine babesiosis in Taiwan. The atovaquone–azithromycin (AA) combination has been suggested to be an effective antibabesial therapy for the treatment of *B. gibsoni* Asian genotype infections since 2004 (Birkenheuer et al., 2004). When this combination was administered in our study (*n* = 17), 9 dogs (52.9%) recovered, 7 dogs (41.2%) relapsed with drug-resistant *B. gibsoni*, and 1 dog exhibited nonremission after treatment. (The mutant strain was detected at time of presentation, with a prior history of AA therapy.) These results are similar to the findings of a smaller study (*n* = 8) by Sakuma et al. (2009), who treated 8 clinically infected dogs for 10 days using the AA combination. Only 2 of the 8 dogs fully recovered, and 5 of the 8 dogs relapsed with drug-resistant *B. gibsoni* (Sakuma et al., 2009). Single or multiple substitutions in the *B. gibsoni* CYTb gene have been associated with atovaquone resistance. A single replacement in nucleotide 363 (G to T/A), which results in the M121I amino acid substitution, has been reported to be the major resistance-inducing mutation (Matsuu et al., 2006; Sakuma et al., 2009). In our study, the M121I mutation was also observed in the AA-relapse and AA-nonremission samples. No other mutations were observed in the sequence from nt 93 to 370. Therefore, the M121I mutation is very likely to be associated with atovaquone resistance. The mean AA administration time in the AA-recovery dogs was 21.7 days, which was longer than the suggested therapy duration. In our study, only 7 of the 17 dogs relapsed with drug-resistant *B. gibsoni* after treatment with the AA combination, whereas 5 of the 8 dogs in the study by Sakuma et al. (2009) relapsed with...
drug-resistant *B. gibsoni* after 10 days of AA combination therapy. Sakuma et al. (2009) found that the 10-day therapy duration may be inadequate for clinically infected dogs. Our findings support the hypothesis that a longer period of AA therapy, with the same protocol that Birkenheuer et al. suggested, could decrease relapse rates.

Drug resistance and high costs are the major disadvantages of the AA combination; these disadvantages underscore the need for alternative therapeutic strategies. Studies have reported several potential *B. gibsoni* treatment combinations, including a combination of clindamycin, doxycycline, and metronidazole (CdDM) (Suzuki et al., 2007). The efficacy of this combination was evaluated in four splenectomized dogs that were experimentally infected with *B. gibsoni*. Three of the 4 dogs recovered after 50 days of treatment. In our CDI combination study, 11 of the 13 dogs recovered after approximately 42 days of therapy, with a recovery rate of 84.6%. Our combination therapy produced a higher recovery rate and required a shorter administration time than the aforementioned CdDM combination therapy. The clindamycin dose in our study was 30 mg/kg body weight, which was higher than the dose that has been used in other experimental studies (25 mg/kg body weight) (Suzuki et al., 2007; Wulansari et al., 2003a,b). Clindamycin is an immune-enhancing antibiotic and has the ability to damage or inactivate *B. gibsoni* (Wulansari et al., 2003a,b). However, Matsu H et al. (2008) found that the activity of clindamycin against *B. gibsoni* was 16–24 times lower than that against *Babesia divergens*. Our study indicates that the antibabesial ability of clindamycin may be dose-dependent. In addition, no clinical side effects were observed in dogs that were orally administrated a dose of 30 mg/kg body weight. We prescribed the traditional antibabesial drugs diminazene acetate and imidocarb diproprionate as the initial therapy; these drugs alone could not eliminate *B. gibsoni* in the affected dogs, but each approach has shown to reduce the severity of the clinical signs and mortality associated with *B. gibsoni* infections (Birkenheuer et al., 1999). In our study, the combination of these two drugs reduced the severity of the disease more quickly. In addition, no adverse effects of diminazene acetate and imidocarb diproprionate administration were observed during therapy. Moreover, the expense of AA combination is 5–6 times more than CDI combination of the entire treatment course when purchased in Taiwan.

The relapse rate was much higher in the AA group (41.2%) than in the CDI group (15.2%). The M121I mutation in the *B. gibsoni* CYTb gene was detected in all of the AA-relapse dogs, whereas no mutations were observed in any of the CDI-relapse dogs. Of the AA-relapse dogs, cases 8 and 10 were switched to the CDI combination treatment when they relapsed on days 203 and 49, respectively. These dogs recovered from anemia and parasitemia 42 and 35 days after the CDI therapy, respectively. Cases 11 and 13 were treated with the AA combination again when they relapsed on days 144 and 56, respectively, but they showed no improvement. Therefore, these two dogs were switched to the CDI combination treatment for another 42 days and have not relapsed at the time of this writing. We conclude that it is very important to detect the M121I mutation to determine the cause of resistance in AA-relapsed cases, and the CDI combination therapy is effective for the treatment of the mutated strain.

There were no differences between the hematological examination results of the two groups on day 0; however, on day 7 of the AA and CDI therapy regimens, the
median Hb, PCV, RBC, and platelet values were significantly higher in the AA group than in the CDI group, especially for the platelet counts. One possible explanation for this phenomenon may be that the major parasiticidal mechanism of atovaquone involves the protozoan CYTb gene and the inhibition of mitochondrial electron transport (Birkenheuer et al., 2004; Fowler et al., 1972; Matsu et al., 2004). In a previous in vitro study, B. giban was found to be susceptible to atovaquone (Matsu et al., 2008); however, in our study, there were no differences in the hematological examination results of the 2 groups on days 14, 21, and 28. Furthermore, the median Hb, PCV, and RBC values were even slightly higher in the CDI group than in the AA group on day 28.

No M121I mutations were detected in the samples that were collected on the day of presentation, except in one AA-nonremission dog that might have been treated with atovaquone five months prior to visiting the NTU VH. This result suggests that, thus far, no M121I mutations in the B. giban CYTb gene have been detected in nature in Taiwan, which implies that the dogs relapsed after AA therapy and were not reinfected. In addition, a point mutation in the 96th nucleotide of the B. giban CYTb gene (from CTG to CTT) resulted in a silent mutation in all of our 22 samples, which differs from Japanese reports (Matsu et al., 2006).

In conclusion, in this study, the M121I mutation in the B. giban CYTb gene was detected in all AA-relapse and AA-nonremission dogs but not in CDI-relapse dogs, which demonstrates that the M121I mutation is associated with atovaquone resistance. Compared to the AA combination, the higher recovery and lower relapse rates (but longer therapy duration and slower reduction in the number of circulating parasites) with the CDI combination indicates that the latter is a more effective treatment for B. giban.

Therefore, the CDI combination is an effective strategy for treating B. gibzoni as an initial therapy and when the M121I gene has mutated.

References


