NOTE *Internal Medicine*

A Possible Treatment Strategy and Clinical Factors to Estimate the Treatment Response in *Bebesia gibsoni* Infection

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ABSTRACT. The effectiveness of combination therapy using clindamycin, metronidazole and doxycycline against canine babesiosis, and the usefulness of platelet count and the plasma C-reactive protein (CRP) concentration as an estimation factor for treatment, were evaluated in four dogs experimentally infected with *Babesia gibsoni*. The combination therapy successfully eliminated *B. gibsoni* in peripheral blood in 3 of 4 dogs, however the remaining dog showed obvious uncontrolled relapse after a temporary recovery. In addition, it was shown that CRP levels decreased in an inverse relationship to the recovery of packed cell volume and therefore CRP levels could be used as an optional clinical marker to estimate the response to treatment.
KEY WORDS: babesia, canine, treatment.

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Canine babesiosis is a tick-borne disease in dogs and both *Babesia gibsoni* and *B. canis* are seen in dogs in Japan [1, 2, 11, 12]. *B. gibsoni* infected dogs are generally seen in the west of Japan and dogs affected by *B. canis* are mainly observed on Okinawa island. However, the incidence of canine babesiosis, especially *B. gibsoni* infection, has been recently reported to be increasing, and the areas affected are expanding towards the northeastern part of Japan [15, 17, 27]. Clinical symptoms of canine babesiosis include anorexia, depression and exercise intolerance as a result of hemolytic anemia. Furthermore, severely affected dogs also often present with disseminated intravascular coagulation (DIC), acute renal failure and immune-mediated hemolytic anemia (IMHA). For these dogs, *B. gibsoni* infection can be a fatal infectious disease. Fatality in babesia-infected dogs is mainly due to the lack of a specific remedy against *B. gibsoni* infection.

Diminazene aceturate (Ganaseg®, NOVARTIS, Tokyo, Japan) has been used for the treatment of *B. gibsoni* infection in Japan until now. However, treatment with diminazene often fails to eliminate *B. gibsoni* from the affected dog, and a relapse of babeisosis is observed at high incidence even after a temporary improvement is obtained by the administration of diminazene. In addition, this therapy is considered to have a narrow margin of safety for dogs and occasionally induces severe adverse effects including hemorrhage in the cerebella, hepatotoxicity and necrosis at the injection site [6, 26]. Because of these finding, a review for alternative therapies has recently been initiated. A treatment protocol, using a combination of the antiprotozoal atovaquone and the antibiotic azithromycin was proposed and showed great effectiveness in treating *B. gibsoni* infection [3, 5, 6, 13]. However, a recent report showed the possible induction of drug resistant mutants of *B. gibsoni* against this treatment protocol [14, 16]. Another candidate for anti-babesia therapy, clindamycin has also been evaluated in the fields of medicine and veterinary medicine [6, 24–27]. Clindamycin is one of the lincomycin antibiotics which has been shown to be effective against human babesiosis [25–27]. However, it is suggested that treatment with clindamycin alone is not enough to eliminate the clinical symptoms and babesia in blood [25–27]. Therefore other therapies, in addition to clindamycin, will be necessary to obtain a satisfactory anti-babesia effect. Previously, one of antitrichomonal, metronidazole, was reported to show therapeutic effect against *B. gibsoni* infection [9]. In addition, one of tetracyclines antibiotics, doxycycline was also shown to have the prophylactic effect on the onset of *B. canis* infection [23]. Although a complete elimination of *B. gibsoni* from infected dogs has not been reported on each drug, it seems that the additive or synergistic effect could be expected if we used these two drugs with clindamycin. In the present study, the therapeutic potential of a combination of these three drugs, which are routinely used in small animal practice, was evaluated in dogs experimentally infected with *B. gibsoni*, in order to establish an effective, less toxic and economical treatment strategy. We also evaluated whether the platelet (PLT) count in peripheral blood, and plasma C-reactive protein (CRP) level, which can be routinely measured in hospitals, would be clinically useful tools to estimate the effectiveness of the therapy, because thrombocytopenia is one of typical laboratory findings at the onset stage of *B. gibsoni* infection and *B. gibsoni* induces hemolysis, a type of tissue necrosis.

Four clinically healthy dogs were used in a series of studies. They were housed and maintained, and all experimental

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procedures were performed in accordance with the Guidelines for Animal Experimentation of Kagoshima University. All the dogs were confirmed to be negative for *B. gibsoni* by polymerase chain reaction (PCR) analysis to detect babesia-derived genome as described below, and showed no abnormal results on complete blood count (CBC) and blood chemistry analyses before the experimental *B. gibsoni* inoculation. Splenectomies were performed in all four dogs before (dogs No. 1–3) or after (dog No. 4) the experimental *B. gibsoni* infection, under anesthesia. *B. gibsoni*-infected erythrocytes were used as an inoculum and were obtained from a dog referred to the veterinary teaching hospital at Kagoshima University for diagnosis and treatment. This dog was given a diagnosis of *B. gibsoni* infection from the findings of a hematological examination (severe hemolytic anemia, detection of *B. gibsoni* in erythrocytes, and thrombocytopenia) and of PCR analysis. Whole blood, which contained the equivalent of 5.0×10^7 *B. gibsoni* particles was used to inoculate the dogs intravenously before or after splenectomy. Collection of blood samples was performed at 2–5 day intervals before and after the inoculation with Babesia-infected blood. EDTA-treated blood specimens were used for CBC, PCR analysis and determination of the infection rate by blood smear. At the same time, half of the collected blood samples were anti-coagulated with heparin and used to prepare plasma for biochemical analyses. CBC was performed using an automatic blood calculator (pocH-100i, Sysmex, Kobe, Japan). Blood smear specimens were stained with modified Wright-Giemsa staining. The infection rate of *B. gibsoni* in erythrocytes was calculated on blood smear specimens. The infection rate was calculated as a percentage of parasite infected erythrocytes/1000 erythrocytes. The remaining EDTA-treated blood samples were stored at –80°C until PCR analysis. Plasma CRP levels were determined using Arrows Laser CRP-2 (Arrows, Osaka, Japan). Detection of *B. gibsoni* derived genomic DNA by PCR was performed as described previously [4, 10]. A single amplification technique and nested PCR method were applied to detect the *B. gibsoni* specific *P18* gene and to amplify the *18S rRNA* gene of the *Babesia* species respectively, as reported by Fukumoto *et al.* and Birkenheuer *et al.* [4, 10].

Diminazene aceturate (Ganaseg®) was subcutaneously administered at a dose of 3 mg/kg, three times when the *B. gibsoni* experimentally inoculated dogs showed less than 15% packed cell volume (PCV). After that, in cases where the dogs showed no improvement of PCV or started a decrease of their PCV, clindamycin (25 mg/kg, PO, BID; Dalacin, Dainippon Sumitomo Pharma, Osaka, Japan), metronidazole (15 mg/kg, PO, BID; Flagyl, Shionogi, Osaka, Japan) and doxycycline (5 mg/kg, PO, BID; Vibramycin, Pfizer, Tokyo, Japan) were administered to dogs No. 1–4 daily. In the present study, "relapse" of the babesiosis was defined as dogs having less than 20% of their PCV after diminazene treatment. A blood transfusion was performed when the dogs showed less than 10% of PCV, anorexia or exercise intolerance during the observation period.

The clinical course of the dogs used in this study is shown in Fig. 1. Briefly, dog No.1 started to show thrombocytopenia at 10 days post inoculation (PI) when the PLT count was lowest ($0.9 \times 10^4/\mu l$). PCV also decreased at 12 days PI and was lowest (10%) at day 18 (Fig. 1A). Plasma CRP concentration (reference range, <1.0 mg/d*l*) started to increase and reached the highest value (4.3 mg/d*l*) at 22 days PI. The administration of diminazene was performed at 15, 18 and 20 days PI, and 250 m*l* of whole blood was transfused at 18 days PI. A temporary improvement in the clinical condition and clinicopathological findings was observed by 20 days PI, however the complete elimination of *B. gibsoni* from peripheral blood could not be obtained. The PLT count and PCV decreased to $7.5 \times 10^4/\mu l$ and 8% at 26 days and 36 days PI, respectively. The infection rate increased to 4.8% at 34 days PI. We judged that the diminazene treatment failed to eliminate *B. gibsoni* and this dog relapsed babeisosis. A blood transfusion was performed at 36 days PI once again and a combination therapy with clindamycin, metronidazole and doxycycline was initiated from 37 days PI. From 10 days after the initiation of this therapy, the dog showed an improvement of clinical symptoms, increase of PCV and a decrease of the CRP concentration. PCV and CRP were normalized by 110 and 40 days PI, respectively. The PLT count became stable by 48 days PI. The disappearance of parasite infected erythrocytes on the blood smear specimen was achieved by 54 days PI. The combination therapy was discontinued at 129 days PI and the PCR result became negative by 160 days PI and any signs of relapse had not been observed by 310 days PI.

Dog No. 2 showed a similar clinical course to that of dog No. 1. A decrease in PLT number had been observed from 6 days PI and become the lowest ($0.6 \times 10^4/\mu l$) at 16 days PI (Fig. 1B). PCV started to decrease from 14 days PI and bottomed (10%) at 17 days PI. The elevation of CRP started from 17 days PI. This dog received diminazene at 17, 19 and 20 days PI, and blood transfusions at 17 and 19 days PI. Although a temporary improvement was observed until 30 days PI, no elimination of parasite infected erythrocytes was observed, and severe thrombocytopenia ($0.7 \times 10^4/\mu l$) and anemia (PCV 8%) developed once again at 34 and 36 days PI, respectively. CRP levels also started to increase again by 34 days PI. This dog showed a relapse of babesiosis judging by the clinical and laboratory findings, and the combination therapy was initiated at 37 days PI in addition to the blood transfusion at 36 days PI. After the initiation of combination therapy, recovery of PCV, PLT count and CRP concentration to the reference range was obtained at 74, 51 and 44 days PI, respectively. *B. gibsoni* in blood smears also disappeared on 48 days PI and was not detected during the remaining observation period. The combination therapy was discontinued at 129 days PI. The PCR result became negative at 201 days PI and any signs of relapse had not been observed by 310 days PI.

In the dog No.3, thrombocytopenia started to develop at 12 days PI and progressed to $0.3 \times 10^4/\mu l$ at 18 days PI (Fig. 1C). The PCV fell to 10% at 19 days PI and CRP also

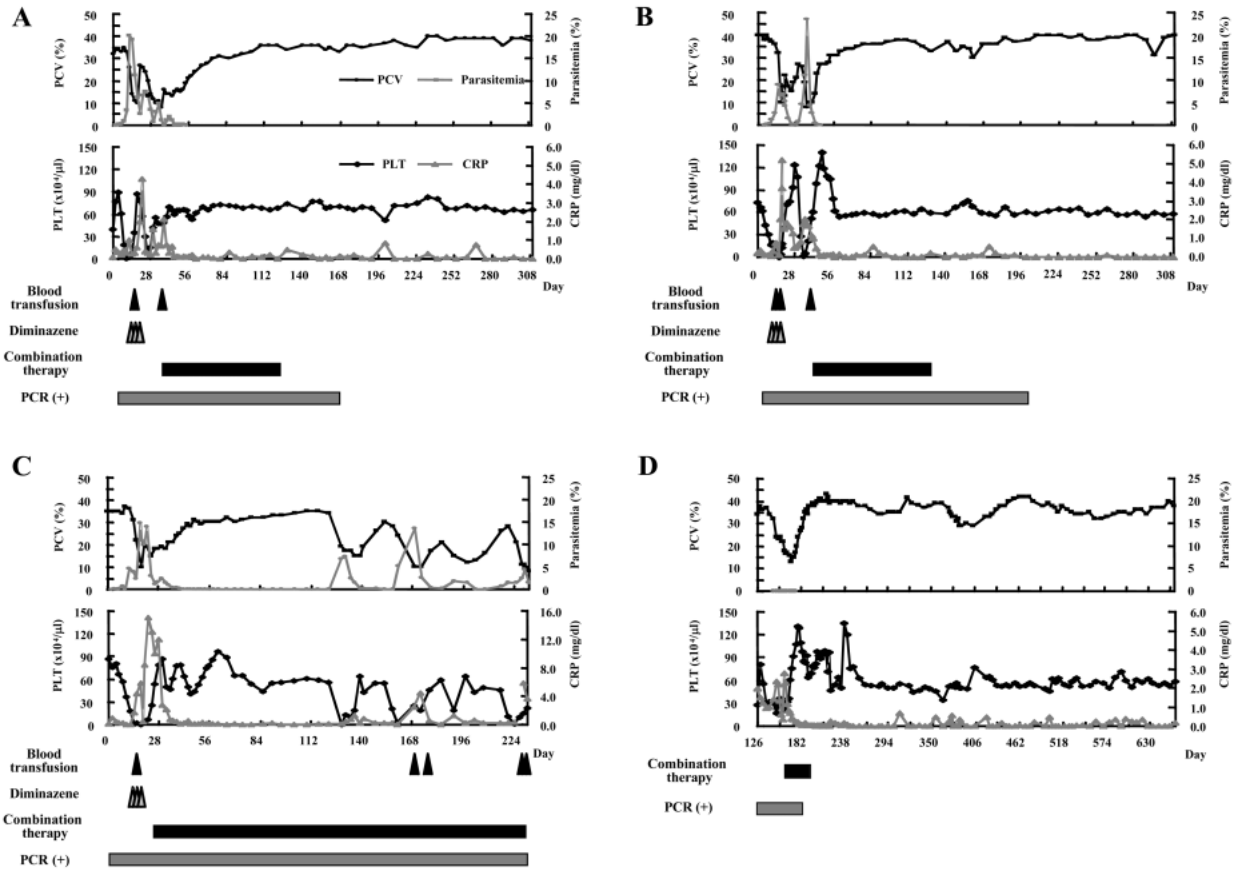

Fig. 1. Changes in clinical parameters and performed treatments. The results derived from each dog are shown in A-D. The dates of blood transfusion and diminazene administrations are indicated as black or grey arrowheads, respectively. The black box represents the duration of combination therapy and the grey box shows the time period for which positive PCR results were obtained. Panels A-D correspond to the results from dogs No. 1–4, respectively.

showed an abnormal value at 14 days PI. Diminazene treatment was given at 18, 19 and 20 days PI and a blood transfusion was performed at 19 days PI. A partial response to the diminazene treatment was observed in this dog, however this treatment failed to eliminate parasite infected erythrocytes from blood or to improve the clinical condition. Because the PLT count and PCV fell to $7.5 \times 10^4/\mu l$ and 15% at 22 and 24 days PI, the combination therapy was initiated at 26 days PI. After the initiation of the combination therapy, PCV and CRP in this dog recovered to reference ranges by 110 and 34 days PI, respectively. Stabilization of the PLT count was obtained by 91 days PI and parasite infected erythrocytes had been eliminated by 42 days PI. However, this dog started to show depression and anorexia at 129 days PI. Laboratory test results on this day revealed severe anemia (PCV 19%), thrombocytopenia ($1.0 \times 10^4/\mu l$) and a high infection rate (6.8%), so a blood transfusion was performed. After that, this dog repeated a partial recovery and relapse, and was euthanized at 232 days PI. PCR analysis showed continuous positive results during the observation period.

Dog No. 4 was inoculated with the *B. gibsoni* infected blood before splenectomy. After the inoculation, *B. gibsoni* detection by blood smear was positive by 4 days PI and the result of PCR analysis became positive next day. However, no clinical signs or clinicopathological abnormality was observed at this point. After that, both test results converted to negative by 21 days PI. A splenectomy was performed on this dog at 126 days PI after which the PLT count and PCV fell to their lowest levels, $16.9 \times 10^4/\mu l$ and 13% at 151 and 169 days PI, respectively (Fig. 1D). Elevation of plasma CRP was observed and coincided with the development of anemia and reached a peak (2.75 mg/d*l*) at 161 days PI. This dog received the combination therapy without diminazene administration from 169 days PI. The PCV recovered to within the normal range by 187 days PI and the PLT count stabilized by 181 days PI. *B. gibsoni* in the blood smear specimens had been detected since 151 days PI, however it disappeared from 175 days PI onwards. In agreement, PCR analysis also showed positive results until 129 days PI and became negative from 181 days PI onwards. Even though the combination therapy was terminated at 193 days PI, this dog never showed any signs of babesiosis by 662 days PI.

To establish a new therapeutic strategy against *B. gibsoni* infection, we evaluated the effectiveness of a combination therapy with clindamycin, metronidazole and doxycycline, which were reported to be useful for canine, human and mouse babesioses [9, 23–27]. As shown in Fig. 1, all the dogs which received the combination therapy showed a recovery of PCV and had no relapse during the treatment or entire observation period, except one case. This therapeutic effect was observed even in dogs which had been treated with diminazene and relapsed. We did not measure the residual concentration of diminazene at the initiation of combination therapy, therefore, it cannot be deny on the synergistic effect of diminazene and combination therapy. However, all of three dogs received diminazene treatment showed relapse or no recovery of PCV and PLT count, we consider the possibility of the synergistic effect unlikely. Although it was known that clindamycin, metronidazole and doxycycline induce pseudomembranous colitis, peripheral neuropathy and hepatopathy, respectively, such adverse effects were not detected in any dogs in this study [7, 19, 21, 22]. Thus, the therapeutic protocol used in this study is a new candidate for the treatment of *B. gibsoni* infection.

However, there are still many problems to be clarified concerning this combination therapy. The first problem is that we did not set the appropriate control dogs to compare the effect of combination therapy. If the dogs receiving combination therapy showed a rapid recovery from anemia and thrombocytopenia or a long disease-free period compared to the untreated control dogs, the undoubted clinical usefulness of this combination therapy would be confirmed. The second problem is that we do not know how each drug eliminates *B. gibsoni in vivo*. Although it was reported that clindamycin based treatments do not have the ability to kill the babesias, further analyses will be required to address each drug's specific pharmacological action against *B. gibsoni* [26]. In addition, we used three drugs in this study, however, it might be an overloaded choice. It will be required to study the possible use of clindamycin with metronidazole or with doxycycline. Recently, the simultaneous use of atovaquone and azithromycin was reported to induce the rapid elimination of *B. gibsoni* [5]. The difference in effect between our combination therapy and the simultaneous use of atovaquone and azithromycin should also be evaluated. The third problem is that our combination therapy took a relatively long time to show clinical effectiveness. Our results indicate, that it took approximately 50 days from the initiation of the combination therapy until an improvement of clinical symptoms and laboratory test were seen. Thus, it can be expected that aggressive supportive care, such as blood transfusion, will be required for severe cases. Namikawa *et al.* reported that the blood transfusion alone showed therapeutic effect on *B. gibsoni* infected dogs [18]. Our cases received a limited number of blood transfusion compared to the report of Namikawa *et al*., however, the importance of blood transfusion should be addressed in future [18]. The forth problem is the duration of medication. In the present study, we administered the three drugs dependent on the clinical status of each dog. Thus, we could not determine the appropriate time point for withdrawal of the medication. The fifth problem is the possibility of relapse after and/or during the combination therapy. One case in this study obviously showed a relapse of babesiosis, even during the combination therapy. Currently, we do not know the difference between the 3 dogs treated successfully and this case. This point should also be clarified from the perspective of immunology, parasitology and pharmacology in the near future.

According to our findings that the combination therapy successfully improved the clinical condition and anemia in dogs No. 1, No. 2 and No. 4, and temporarily in No. 3, we could evaluate the usefulness of the clinical estimation factors in measuring the response to treatment. We selected two parameters, PLT and CRP, because they are routinely measured in hospital, and we analyzed the relationships between PCV and these parameters using Pearson's correlation coefficient in recovery phase. In the present study, the recovery phase was defined from the day that the combination therapy was initiated until the day that PCV became stable (Dog No. 1, 37–110 days PI; Dog No. 2, 37–74 days PI; Dog No. 3, 26–110 days PI; Dog No. 4, 169–187 days PI). A significant relationship between the recovery of PLT and PCV during the recovery phase was observed in two dogs but not in the others (Fig. 2). A significant inverse relationship between plasma CRP concentrations and the recovery of PCV was detected in all dogs (Fig. 3). Therefore, CRP seemed to be one of candidate with which to gauge the effectiveness of the therapy. Canine CRP is known to be a very sensitive parameter to detect the inflammatory lesion or necrotic events *in vivo* [8, 20]. In our experiences in clinics, most of cases with *B. gibsoni* infection reveal high concentration of CRP level even though the affected dogs show no obvious clinical signs or parasitemia (data not shown). It might be possible to detect small amount of hemolysis without anemia on hematological examination or detectable parasitemia. PCR is very sensitive method to detect *B. gibsoni*, however, it cannot be performed routinely in hospital other than a commercial or research laboratory because of its cost and tangled procedures. Therefore, it will be important to see the alteration of CRP levels with PCV. However, the usefulness of these parameters was limited to the recovery phase or treatment period from the findings in this study. In one case (dog No. 3), a second relapse and resistance to the combination therapy was seen, even though this dog had showed an ideal response to the combination therapy until 97 days after the initiation of therapy (Fig. 1C). Because of this finding, the recovery of CRP and PLT values are not recommended as estimates of the prognosis.

In summary, we propose a new therapeutic strategy and estimation factors for the treatment of *B. gibsoni* infection in dogs, which is a very important infectious disease in small animal practice. The findings obtained in this study seemed to give useful information about the control of canine babesiosis. This work was supported by grants from the Japanese Society for the Promotion of Science.

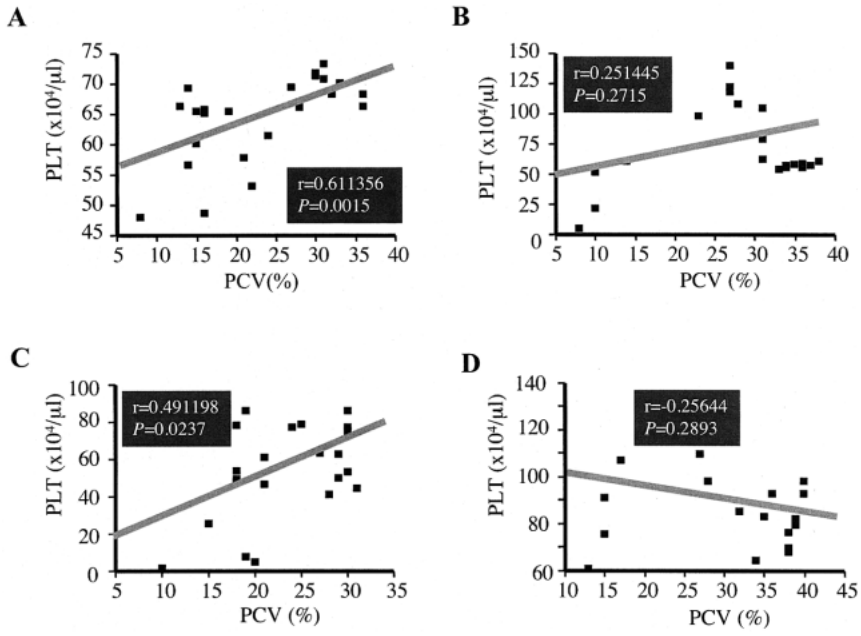

Fig. 2. Correlation coefficient between PCV and PLT counts in four dogs treated with the combination therapy. Panels A-D correspond to the results from dogs No. 1–4, respectively.

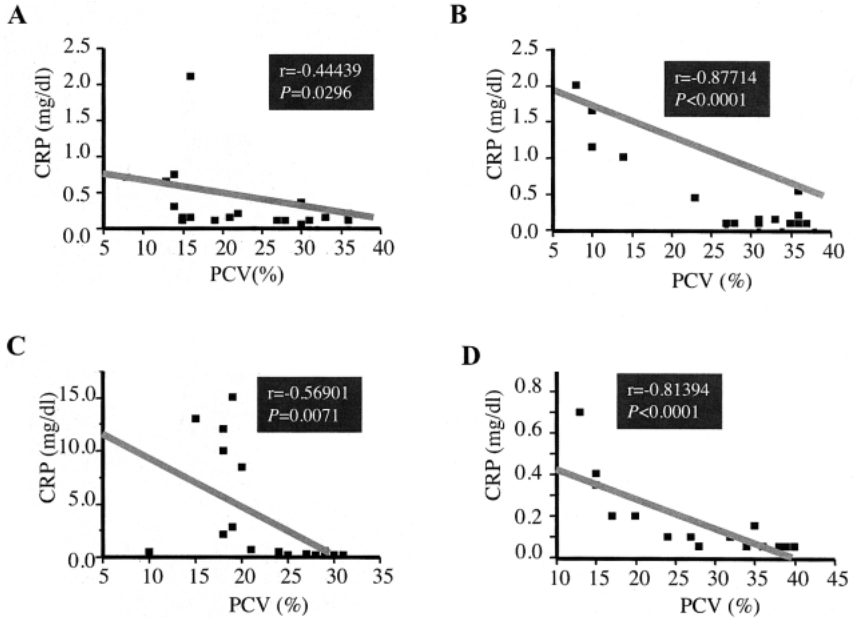

Fig. 3. Correlation coefficient between PCV and plasma CRP levels in four dogs treated with the combination therapy. Panels A-D correspond to the results from dogs No. 1–4, respectively.

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