

## In Vitro Evaluation of Drug Susceptibilities of *Babesia divergens* Isolates

PHILIPPE BRASSEUR,<sup>1\*</sup> SOPHIE LECOUBLET,<sup>1</sup> NATHALIE KAPEL,<sup>2</sup>  
LOIC FAVENNEC,<sup>1</sup> AND JEAN J. BALLE<sup>3</sup>

Laboratoire de Parasitologie, Centre Hospitalier Universitaire, Hôpital Charles Nicolle,  
76031 Rouen,<sup>1</sup> Laboratoire de Parasitologie, Faculté de Sciences Pharmaceutiques  
et Biologiques, 75006 Paris,<sup>2</sup> and Laboratoire d'Immunologie et  
Immunopathologie, Centre Hospitalier Universitaire,  
Hôpital Clémenceau, 14033 Caen,<sup>3</sup> France

Received 18 August 1997/Returned for modification 31 October 1997/Accepted 27 January 1998

**The susceptibilities of three bovine and two human *Babesia divergens* isolates to antimicrobial agents were evaluated in vitro by a tritiated hypoxanthine incorporation assay. The MICs at which 50% of isolates are inhibited (MIC<sub>50</sub>s) for mefloquine (chlorhydrate), chloroquine (sulfate), quinine (chlorhydrate), clindamycin (phosphate), pentamidine (isethionate), phenamidine (isethionate) plus oxomemazine (chlorhydrate), lincocmycin (chlorhydrate monohydrate), and imidocarb (dipropionate) were determined. Except for imidocarb, the MIC<sub>50</sub>s observed for the different isolates were close. Imidocarb and the combination of phenamidine plus oxomemazine exhibited the highest in vitro activity, while antimalarial agents such as mefloquine, chloroquine, and quinine were inactive. Other drugs had intermediate activities. The data support further in vitro evaluation of antimicrobial agents active against *B. divergens* for the improvement of therapeutic strategies.**

Babesiosis is due to an intraerythrocytic protozoan parasite transmitted by tick bites to a wide variety of wild and domestic animals, including cattle, horses, dogs, and rodents. In Europe, *Babesia divergens* is considered the most pathogenic agent of bovine babesiosis and is responsible for important economic losses in beef- and milk-producing breeds. Current therapy includes the use of imidocarb dipropionate, but it remains debatable whether differences in outcomes among individual animals are due to variations in the sensitivities of the isolates to drugs or to host factors in animals which are not highly inbred (11).

In humans, 19 of the 24 cases of babesiosis reported in Europe were due to *B. divergens*, and all occurred in splenectomized patients. The initial therapeutic trials with antimalarial drugs for the treatment of this condition, characterized by severe symptoms and high mortality rates, have had limited success. More recently, complete parasite clearance and recovery were obtained with the combination of clindamycin and quinine in association with exchange transfusion (6).

Limited information is available on the antimicrobial susceptibility of *Babesia* spp. in vitro. The first reports of the use of tritiated nucleic acid precursors for the screening of drugs (7) showed a positive correlation between in vitro and in vivo activities in animals (10). More recently, in vitro assays have been proposed for determination of the susceptibilities of *Babesia bovis* and *Babesia bigemina* to drugs (4, 12). The aim of this work was to compare the antimicrobial susceptibilities of *B. divergens* isolates of bovine and human origin by a tritiated hypoxanthine incorporation assay.

### MATERIALS AND METHODS

***B. divergens* isolates.** Five *B. divergens* isolates were tested. Three isolates were of bovine origin and were obtained from different geographical areas in Europe:

Muenchen (Munich, Germany), Weybridge 8843 (Weybridge, United Kingdom), and Taylor (Ireland). Two isolates were obtained in France from patients admitted to Rouen University (isolate Rouen) and Le Mans (isolate Le Mans) hospitals. Isolates were maintained in human erythrocyte (RBC) cultures and infected 10 to 20% of the RBCs. The isolates were then suspended in RPMI 1640 medium (Gibco, Cergy, France) supplemented with 40% (vol/vol) fetal calf serum and 15% (vol/vol) dimethyl sulfoxide and frozen in liquid nitrogen.

***B. divergens* cultures.** After thawing and washing, 0.2 ml of packed infected RBCs was injected intraperitoneally into 2-month-old female gerbils (*Meriones unguiculatus*) (Centre D'élevage R. Janvier, Le Genest-Saint-Isle, France) 2 days after an intraperitoneal injection of 2 mg of dexamethasone (Soludécadron; Merck-Sharp-Dohme, Paris, France). When the level of parasitemia reached 50% (i.e., 2 to 3 days later), parasitized RBCs were collected by cardiac puncture. Human RBCs were collected from healthy type O- and Rh-positive volunteer blood donors, placed in preservative-free heparin (Heparine Leo, Saint-Quentin-Les-Yvelines, France), and washed by serial centrifugations in RPMI 1640 medium. The buffy coat and the upper part of the RBC pellets were discarded to avoid leukocyte contamination, and 0.2 ml of packed human RBCs was poured into 50-ml plastic flasks (Nunc, Roskilde, Denmark) containing 5 ml of RPMI 1640 medium supplemented with HEPES (25 mM; pH 7.4; Sigma, St. Louis, Mo.), gentamicin (12 µg/ml), and 10% (vol/vol) decomplexed pooled human serum from type O- and Rh-positive donors. A volume of 0.01 ml of parasitized packed gerbil RBCs was added. The cultures were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere and were supplemented with human RBCs twice a week to adjust the level of parasitemia to between 1 and 2%.

**In vitro assays for drug susceptibility.** Drug susceptibility assays were performed in 96-well culture microplates (Falcon, Meylan, France) in the presence of a final RBC concentration of 1.8% (vol/vol) and serial dilutions of drugs. Each duplicate well received 200 µl of an RBC suspension and 50 µl of each drug dilution in RPMI 1640 medium. For each experiment, controls consisted of four wells containing infected RBCs without drug (i.e., wells to which 50 µl of RPMI 1640 alone was added) and two wells containing 200 µl of noninfected RBCs.

[<sup>3</sup>H]hypoxanthine (specific activity, 4 Ci/mmol; Amersham, Les Ulis, France) was added (0.4 µCi/well). The plates were incubated at 37°C for 48 h in a 5% CO<sub>2</sub> atmosphere, the cells were harvested on glass fiber filters (Whatman GF/C) with a cell harvester, and the incorporated radioactivity (in counts per minute) was counted in a liquid scintillation spectrometer.

Five milliliters of RBCs from each well were stained with Giemsa stain, and 5,000 RBCs were microscopically examined to determine morphologically the percentage of infected RBCs.

**Antimicrobial agents.** From a stock solution in 70% ethanol, nine dilutions of the following antimicrobial agents were prepared in RPMI 1640 medium: mefloquine chlorhydrate (0.3 to 0.017 µg/ml; Hoffmann-LaRoche-Basle, Basel, Switzerland), chloroquine sulfate (6 to 0.023 µg/ml; Rhône Poulenc, Paris, France), quinine chlorhydrate (1.2 to 0.047 µg/ml; Assistance Publique, Paris, France), clindamycin phosphate (250 to 0.98 µg/ml; Upjohn, Val de Reuil, France), pentamidine isethionate (10 to 0.07 µg/ml; Rhône Mérieux, Toulouse, France),

\* Corresponding author. Mailing address: Laboratoire de Parasitologie, Hôpital Charles Nicolle, 1, rue de Germont, 76031 Rouen, France. Phone: (33) 2 32-88-80-15. Fax: (33) 2 32-88-80-17. E-mail: philippe.brasseur@wanadoo.fr.

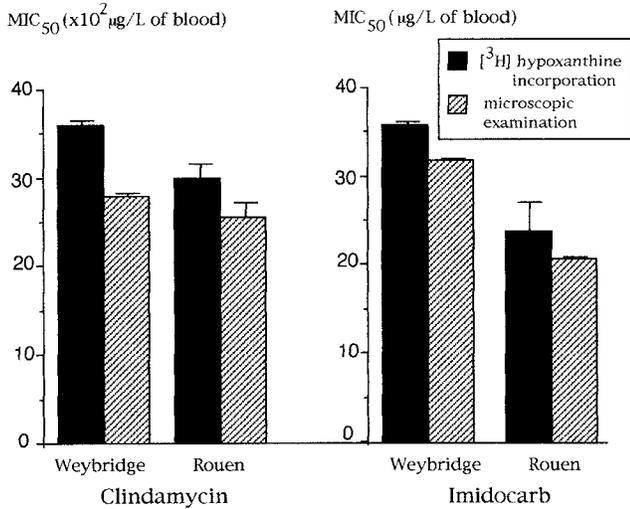


FIG. 1. Comparison of MIC<sub>50</sub>s of clindamycin and imidocarb for the Weybridge 8843 bovine isolate and the Rouen human isolate obtained in the [<sup>3</sup>H]hypoxanthine incorporation assay and by microscopic examination. Results are expressed as mean values for 15 independent experiments (one bar indicates 1 SD).

phenamidine isethionate and oxomemazine chlorhydrate (2 to 0.008 µg/ml; Oxopirvédine; Rhône Mérieux, Lyon, France), lincomycin chlorhydrate monohydrate (4,000 to 15.6 µg/ml; Lincocine; Upjohn, Val de Reuil, France), and imidocarb dipropionate (25 to 0.098 µg/ml; Carbesia; Pitman-Moore, Meaux, France). A volume of 50 µl of each dilution was added to the wells. Control wells were filled with 50 µl of medium without drug.

**Expression of results and statistical analysis.** The amount of parasite-dependent tritiated hypoxanthine incorporated was obtained by subtracting the amount of background incorporation observed in noninfected cultures. For each drug, a concentration-versus-[<sup>3</sup>H]hypoxanthine incorporation curve was established, and the inhibitory activity was expressed as the minimum concentration (wt/vol) of drug which either inhibited tritiated hypoxanthine incorporation or decreased the proportion of morphologically infected RBCs by 50% of the value in control cultures without drug (the concentration at which 50% of isolates are inhibited [MIC<sub>50</sub>]). MIC<sub>50</sub>s were expressed in micrograms per liter of blood with a 50% hematocrit.

Variance analysis was performed by the Fisher and Yates chi-square test or the Student *t* test, thus assuming normal, like distributions of values. The significance of *r* values was used to study linear correlation.

**RESULTS**

In preliminary experiments, tritiated hypoxanthine incorporation was measured in cultures maintained in the absence of antimicrobial agents (15 microplates, 24 wells/microplate). For the noninfected wells, the mean ± 1 standard deviation (SD) level of incorporation was 191 ± 52 cpm/well, and for infected wells without drug, the mean ± 1 SD level of incorporation was 11,707 ± 2,575 cpm/well. No significant difference was found between isolates (*P* > 0.1; data not shown). One SD accounted for less than 5 and 17% of the mean for each microplate and for the pooled data for the 15 microplates, respectively. The variations for the cultures maintained in the presence of the different antimicrobial agents are summarized in Fig. 1 to 3. In the case of clindamycin, it was verified with the Le Mans isolate that a good correlation was found between MIC<sub>50</sub>s obtained with 18 independent microplates (*r* = 0.7; *P* < 0.01).

A detailed comparison of the MIC<sub>50</sub>s obtained by the tritiated hypoxanthine incorporation assay and microscopic counting was performed for the Weybridge 8843 and Rouen isolates in the presence of clindamycin or imidocarb. As indicated in Fig. 1, no significant difference in the MIC<sub>50</sub>s was observed in 15 independent experiments (*P* > 0.1). This was confirmed for

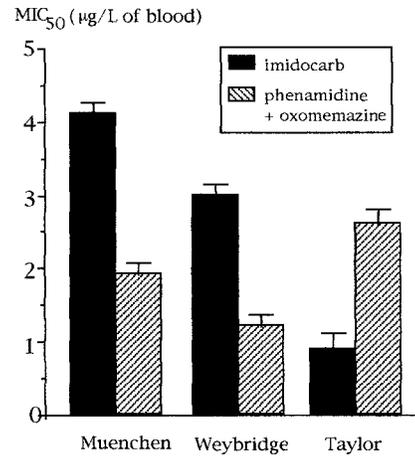


FIG. 2. MIC<sub>50</sub>s of phenamidine plus oxomemazine and of imidocarb for bovine *B. divergens* isolates. Results are expressed as mean values for eight independent experiments (one bar indicates 1 SD).

all isolates in the presence of the other antimicrobial agents (*P* > 0.1; data not shown).

From these data, the MIC<sub>50</sub>s of clindamycin and imidocarb varied from 2,200 to 3,400 µg/liter and from 27 to 34 µg/liter, respectively. Imidocarb, a drug restricted to the treatment of babesiosis in animals, was efficient against both human and bovine *B. divergens* isolates (Fig. 1 and 2). The MIC<sub>50</sub>s of imidocarb and clindamycin for bovine and human isolates were not significantly different; however, MIC<sub>99</sub>s determined by using probit/log concentration regression curves suggested that they had higher levels of activity against human isolates than against bovine isolates (14,100 and 28,800 µg/liter, respectively). The combination of phenamidine plus oxomemazine was more effective than imidocarb against the bovine isolates Muenchen and Weybridge 8843 but not against the bovine isolate Taylor (Fig. 3). None of the *B. divergens* isolates tested was found to be sensitive to antimalarial agents (Table 1). Pentamidine isethionate inhibited *B. divergens* in vitro, and lincomycin was active and had a high MIC<sub>50</sub> (Table 1).

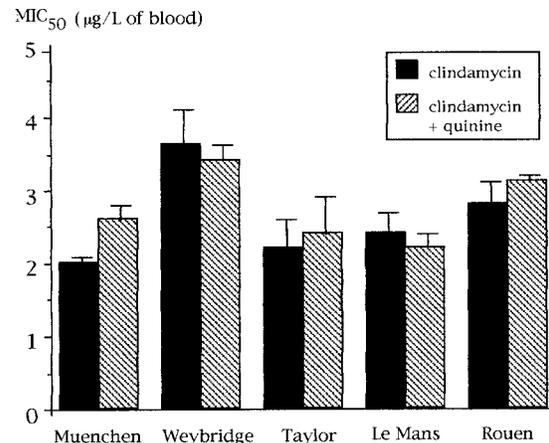


FIG. 3. MIC<sub>50</sub>s of clindamycin and clindamycin plus quinine for bovine and human isolates of *B. divergens* in 18 independent experiments. Results are expressed as mean values for 18 independent experiments (one bar indicates 1 SD).

TABLE 1. In vitro activities of mefloquine, chloroquine, quinine, pentamidine, and lincomycin against bovine and human *B. divergens* isolates

Isolate source and isolate	MIC ( $\mu\text{g/liter}$ of blood) <sup>a</sup>				
	Mefloquine	Chloroquine	Quinine	Pentamidine	Lincomycin
Bovine isolates					
Muenchen	>24	>48	>96	5.1 $\pm$ 1	70,000 $\pm$ 2,100
Weybridge 8843	ND	ND	>96	ND	ND
Taylor	>24	>48	>96	2.5 $\pm$ 0.4	32,000 $\pm$ 3,800
Human isolates					
Le Mans	>24	>48	>96	2.2 $\pm$ 0.0	37,000 $\pm$ 4,700
Rouen	ND	ND	>96	ND	38,000 $\pm$ 2,500

<sup>a</sup> Results are for two to six triplicate experiments. ND, not determined.

## DISCUSSION

In this report a 48-h in vitro assay was used to evaluate the susceptibilities of human and bovine *B. divergens* isolates to antimicrobial agents. Continuous cultures of *B. divergens* were maintained as described previously (7), and the level of incorporation of tritiated hypoxanthine was measured in the presence of different concentrations of drugs. This assay was easier to perform than microscopic counting and was found to be reliable and sensitive. The MIC<sub>50</sub>s did not differ significantly from the values obtained by microscopic examination. Uptake of tritiated purines such as hypoxanthine was previously proposed for the screening of drug activities against *Babesia* spp. with short-term cultures of blood from experimentally infected animals (8, 9). In the present culture assay, which uses human instead of bovine RBCs, higher levels of tritiated hypoxanthine incorporation were obtained.

Bovine babesiosis is generally treated with imidocarb dipropionate. For bovine isolates, the MIC<sub>50</sub> of this compound was significantly lower than the maximum ( $C_{\text{max}}$ ) concentration in serum of 6.6 mg/liter observed in cattle receiving a dose of 17 mg/100 kg of body weight. This is consistent with the high level of activity of imidocarb against bovine babesiosis. Similar MIC<sub>50</sub>s, i.e., 0.87  $\mu\text{g/ml}$  of medium, have been reported for *B. bovis* (15). In addition, the comparable results obtained for human and bovine isolates suggest that imidocarb is possibly a candidate agent for the treatment of human *B. divergens* babesiosis.

The combination of phenamidine isethionate plus oxememazine chlorhydrate, which is currently restricted for use in the treatment of canine babesiosis, was found to be more active than imidocarb in vitro.

The treatment of human *B. divergens* babesiosis with antimicrobial drugs has had limited success. Of the seven patients treated with chloroquine for several weeks, four died and three recovered (1, 5, 10, 14). A treatment based on blood exchange and the administration of clindamycin plus quinine has been successfully used in six patients. Our results confirm the susceptibility of *B. divergens* to clindamycin in vitro. The in vivo efficacy of quinine is controversial (3), since some of the patients treated with quinine alone died and others recovered after several weeks. The very high MIC<sub>50</sub>s of quinine found in this study suggest that this compound is not useful for treatment. The MIC<sub>50</sub>s of antimalarial drugs found in this study were higher than the cutoff limits of sensitivity for *Plasmodium falciparum* (8.6, 2.7, and 1  $\mu\text{g/liter}$  of blood for quinine, chloroquine, and mefloquine, respectively). Interestingly, it has been shown in a recent study that atovaquone, which has anti-

malarial activity, was more active than imidocarb for the treatment of bovine *B. divergens* babesiosis (13).

All isolates were susceptible to pentamidine isethionate in vitro. For the Rouen isolate, the MIC<sub>50</sub> (2.2  $\pm$  0.08  $\mu\text{g/liter}$  of blood) was higher than the  $C_{\text{max}}$  (0.2 to 0.5  $\mu\text{g/liter}$  of blood) resulting from an injection of 4 mg/kg, which is the maximum dose that can be used in humans due to the renal toxicity of this compound (16). This may explain the treatment failures reported for patients treated with pentamidine alone (17) or with pentamidine and chloroquine (2). No data on the pharmacokinetics of pentamidine isethionate in cattle are available, and it cannot be stated whether pentamidine isethionate can be proposed for use in the treatment of bovine babesiosis. The MIC<sub>50</sub> of lincomycin found in this study is higher than the  $C_{\text{max}}$  values obtained in humans (2 to 7  $\mu\text{g/liter}$  of blood), which suggests that lincomycin probably has limited use for the treatment of this disease.

Data suggest that tritiated hypoxanthine incorporation by *B. divergens* in human RBC cultures provides an accurate and convenient means of evaluating the antibabesial activities of drugs, further drug screening, detecting resistant isolates, and comparing in vitro activities with in vivo activities.

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