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Development and Evaluation of a Blood-Free Medium for Determining Growth Curves and Optimizing Growth of *Rochalimaea henselae*

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Two strains of *Rochalimaea henselae* were used to optimize a blood-free growth medium. Seven agar bases, four broths, and combinations of eight supplements were evaluated. Acceptable growth was achieved in media containing Fildes solution and hemin, with the best growth demonstrated in brucella broth or on brucella agar with 6 to 8% Fildes solution and 250 μg of hemin per ml. *R. henselae* utilized hemin in concentrations six times that utilized by *Rochalimaea quintana*. Erythrocyte membrane was necessary to achieve the full growth-promoting effect of rabbit blood.

Over the past 3 years, we have learned a great deal about the clinical significance of infections with members of the genus *Rochalimaea*. These organisms, grouped in the family *Rickettsiaceae*, are now known to be associated not only with trench fever but also with bacillary angiomatosis, parenchymal bacillary peliosis, fever with bacteremia, aseptic meningitis, infective endocarditis, and cat scratch disease. An increasing number of strains of *Rochalimaea henselae* have been isolated recently from patients with these clinical conditions and from human immunodeficiency virus-positive and other immunocompromised individuals as well as from immunocompetent patients (2, 5, 6). A fastidious organism that appeared to be difficult to isolate and grow, it was first detected in tissue by Relman et al. by a polymerase chain reaction technique (3).

Like its most closely related species, *Rochalimaea quintana*, *R. henselae* appears to have stringent nutritional requirements, most notably the demand for an erythrocyte component (1). All of the isolates reported recently have been grown on either chocolate agar or a blood-containing agar, with the best growth reported on 5% rabbit blood agar (6).

Physiological and nutritional studies are greatly facilitated by the ability to grow an organism on defined cell-free medium. Our objective was to develop a medium that would allow optical density (OD) measurements of growth in broth and that would serve as a basis from which to develop media that might facilitate the isolation of *R. henselae* from specimens from patients.

(Some of this material was presented at the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract 373 [4].)

MATERIALS AND METHODS

Bacterial strains. Two separate isolates of *R. henselae* were acquired. One was provided by J. Friero, VA Medical Center, San Diego, Calif.; the other was the Houston-1 isolate kindly provided by Russell Regnery, Centers for Disease Control, Atlanta, Ga. *R. quintana* ATCC VR 358, *Bartonella bacilliformis* ATCC 35685, and *Haemophilus influenzae* ATCC 511 were maintained for growth comparisons and controls. Stock organisms were maintained on either Trypticase soy agar with 5% sheep or 5% rabbit blood (Becton Dickinson Microbiological Systems [BBL], Cockeysville, Md.) or chocolate agar (BBL) incubated at 35 to 36°C in 5% CO_2. Sheep blood and rabbit blood were used interchangeably for routine cultures. On occasion, broths containing 5 to 8% blood were used to enhance initial growth for a period of up to 24 to 48 h.

Preparation of media. Agar and broth bases were prepared according to manufacturers' instructions, aliquoted into screw-top culture tubes (20 by 125 mm; 18 ml per tube), and autoclaved. Supplements were added to tubes cooled to at least 56°C. The growth supplement stock solutions, hemin (5 mg/ml; Eastman Kodak Co., Rochester, N.Y.), cysteine (10 mM solution; Sigma Chemical Co., St. Louis, Mo.), pyridoxal (10 mM aqueous; Sigma), yeast extract (BBL), lysed erythrocyte supernatants, and Fildes solution (BBL), were filter sterilized (0.45-μm-pore-size nitrocellulose filter; MSI, Westboro, Mass.). Fetal calf serum (HyClone Laboratories, Inc., Logan, Utah) was received sterile. Sterile defibrinated rabbit blood (Remel, Lenexa, Kans.) was added to broth to achieve a 5% concentration or suspended in an appropriate volume of sterile water to create the lysed erythrocyte supernatant. Supplements were tested at the following concentrations: cysteine, 0.1 mM final concentration; Fildes solution, 5 and 8%, hemin, 40, 125, 250, and 500 μg/ml; yeast extract, 5%; fetal calf serum, 10%; rabbit erythrocytes, 5%; lysed erythrocyte suspension, 5%. Each supplement was tested individually and in combinations with broths or agars.

The broths tested were heart infusion (Difco Laboratories, Detroit, Mich.), brain heart infusion (Difco), Columbia (BBL), and brucella (BBL). An uninoculated supplemented broth was used as the blank for OD measurements. Agar bases tested included Noble (BBL), proteose peptone no. 3 (BBL), and Fastidious Anaerobe (Lab M, Topley House, London, England). Agar (1.6%; Bacto Agar; Difco) was added to broth bases to make heart infusion, brain heart

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infusion, Columbia, and brucella agars. The broth pH was adjusted aseptically with NaOH or HCl after preparation. Broth and agar bases without supplements were run as controls.

Growth curves. Organisms swabbed from the surface of a 5- to 7-day-old subculture on agar were suspended in Trypticase soy broth (BBL), and the turbidity was adjusted to an OD of 0.65 to 0.95 at 600 nm with a Bausch & Lomb Spectronic 20 apparatus (McFarland standard of approximately 1.0). From this suspension, 0.5 ml (approximately 7.5 $\times$ 10^5 CFU) was inoculated into 10 ml of each broth and supplement to be tested. For determination of CFU, brucella broth with 5% Fildes solution and 250 μg of hemin per ml was used. The broths were incubated at 36°C in 5% CO_2 for 7 days, with intermittent shaking.

Aliquots were removed at intervals for determining ODs and CFUs. To achieve sufficient light transmission for on-scale ODs of the growth medium used for the CFU determination assay, which contained Fildes solution and hemin, aliquots had to be diluted 1:2 in sterile water. All readings were performed in duplicate with two separate aliquots. Uninoculated, supplemented brucella broth base diluted in the same manner was used as the blank. Thus, for this assay, final ODs did not correlate directly to CFUs but the relative values were consistent and correlative. Aliquots were examined microscopically with a Neubauer counting chamber to obtain rough estimates of relative numbers of organisms present, and serial twofold dilutions were prepared in Trypticase soy broth to yield final CFUs of 30 to 300 per plate. Freshly prepared plates (brucella agar with 5% Fildes solution and 250 μg of hemin per ml) were inoculated in duplicate with 0.1 ml of the appropriate dilution, the inocula were spread with glass spreaders, and the plates were incubated as described above for 7 days before counting. Periodic and final subcultures were made onto Trypticase soy-blood agar to verify the lack of contamination. The San Diego and Houston-1 strains were run in parallel and found to behave identically. The figures show readings for the Houston-1 isolate.

Inoculation procedure for comparisons of agar bases. Organism suspensions in Trypticase soy broth were prepared as described above. A sterile cotton swab inserted into the broth suspension and squeezed to remove excess moisture was used to inoculate one quadrant; four organisms were thus tested on a single agar plate. The growth was graded by examining plates under a stereomicroscope. Minimal growth with colonies not visible to the naked eye was graded ±, limited growth with colonies visible to the naked eye was graded 1+, moderate growth was graded 2+, and luxuriant growth filling the entire area was graded 3+.

RESULTS

Brucella agar best supported the growth of *R. henselae* to the degree that brucella broth did (Fig. 1). That brucella agar best supported the growth of *R. henselae* to the degree that brucella broth did was observed throughout the study and was supported by the consistently higher OD readings at plateau phase achieved with brucella broth. Subsequent evaluations of supplements were carried out with brucella broth base. Although Fildes solution alone was sufficient to allow a slow rate of growth, the addition of hemin resulted in enhanced growth as evidenced by a 300% increase in OD (Fig. 2). The growth of *R. henselae* was stimulated by hemin at concentrations of 250 μg/ml (Fig. 3). Neither lysed rabbit blood supernatant (Fig. 4) nor cysteine (data not shown) enhanced the growth of *R. henselae*. There was no difference in growth at pH 6.5 versus pH 7.3. The addition of fetal calf serum or yeast extract did not improve growth. Although previous work with *R. quintana* (1) revealed a requirement for serum in the medium when crystalline hemoglobin was substituted for actual erythrocytes, this requirement was not seen with *R. henselae*. Our results differed from those reported for *R. quintana* in regard to the concentration of hemin tolerated by the organism. Whereas *R. quintana* growth was best at a hemin concentration of 40 μg/ml, our *R. henselae* strains thrived at concentrations

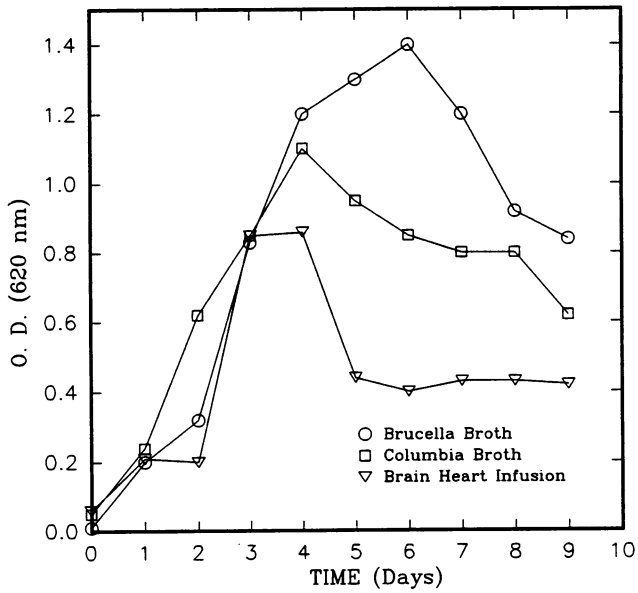

FIG. 1. Comparison of broths for growth of *R. henselae*. Supplements used were hemin (250 μg/ml) and Fildes solution (8%).

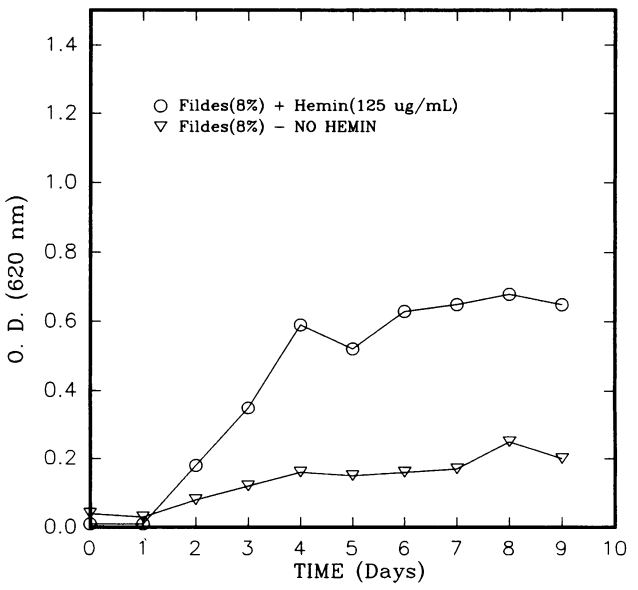

FIG. 2. Additive effects of Fildes solution and hemin in supporting the growth of *R. henselae* in brucella broth.

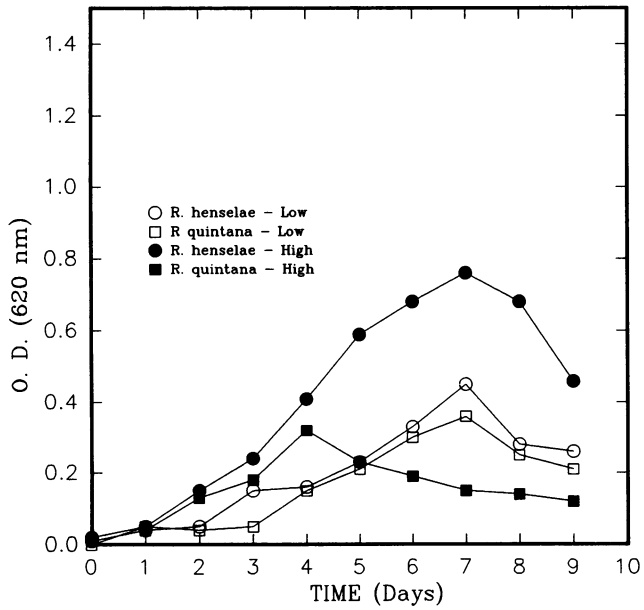

FIG. 3. Differences in hemin utilization between *R. henselae* and *R. quintana*. Low (concentration) = 40 μg/ml; high (concentration) = 250 μg/ml.

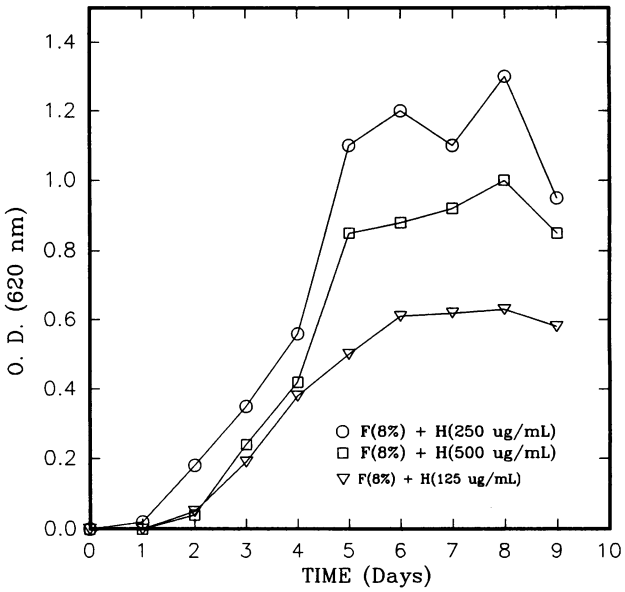

FIG. 5. Effect of hemin concentration on growth of *R. henselae*. F, Fildes solution; H, hemin.

more than six times that level (Fig. 3). Hemin concentrations above 250 μg/ml, however, were found to result in decreased growth, with definite toxicity evident at 500 μg of hemin per ml (Fig. 5).

No agar base without added supplements supported growth rated >± for either of the *Rochalimaea* spp. tested. In the presence of 250 μg of hemin per ml and 5% Fildes solution, the agar bases tested supported growth which was scored from 1+ to 3+. No growth was observed on proteose peptone no. 3 or Fastidious Anaerobe agar, slight growth (1+) was observed on Noble agar, adequate growth (2+) was observed on heart infusion and brain heart infusion, and the best growth (3+) was observed on Columbia and brucella bases with Bacto Agar. The growth in the presence of 5% rabbit erythrocytes was approximately the same as that on agar with Fildes solution and hemin.

Growth curves revealed a slow logarithmic growth phase that lasted for approximately 7 days. Visual counts obtained in the counting chamber closely agreed with those obtained by CFU determination.

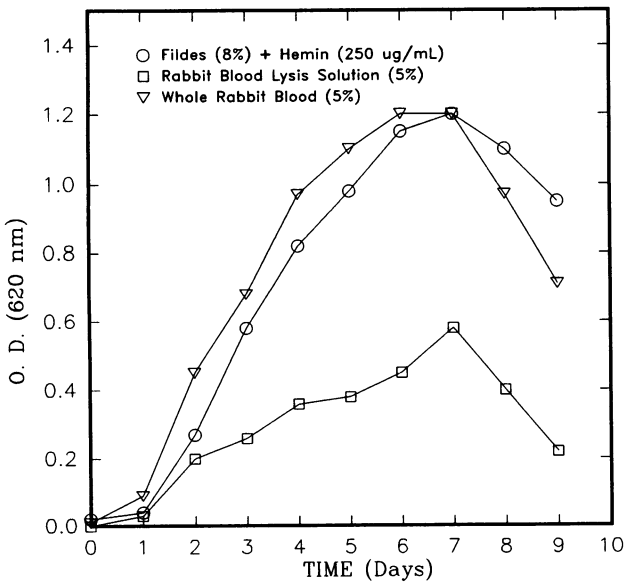

FIG. 4. Effects of whole erythrocytes and lysis supernatant on growth of *R. henselae*.

DISCUSSION

This study has demonstrated that a transparent, blood-free medium containing blood-based components can support growth of *R. henselae* adequate to allow direct OD measurements. We generated growth curves that should be useful as baselines for future studies of physiological characteristics. We noted that the growth-enhancing effect of whole rabbit blood was equal to that of Fildes solution and hemin at optimum concentrations, an effect dramatically reduced with the substitution of rabbit blood lysate without erythrocyte membranes for whole rabbit blood. It is possible that a component of the rabbit erythrocyte cell membrane is utilized for growth. While the clinical profile of *R. henselae* indicates that it may have an intracellular growth phase, we were unable to demonstrate enhanced growth with added cysteine or with a lowered pH. Supplements other than those tested in this preliminary study should be evaluated for the ability to enhance the growth of this fastidious organism. The demonstration of growth of *R. henselae* in the transparent, semidefined medium described here should facilitate such studies. Auxotrophy can play an important role in optimizing an organism's ability to grow in the presence of limiting nutrients. If key precursors for *R. henselae* can be identified, the potential to tailor media that will reduce incubation time and enhance the recovery of this species from patient specimens will exist.

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