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Isolation of *Bartonella* (*Rochalimaea*) *henselae*: Effects of Methods of Blood Collection and Handling

SUE ANNE BRENNER,[1]* JANE A. ROONEY,[2] PABLO MANZEWITSCH,[3] AND RUSSELL L. REGNERY[3]

Division of Infectious Diseases, Emory University School of Medicine,[1] and Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention,[3] Atlanta, Georgia, and HESKA Corporation, Fort Collins, Colorado[2]

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***Bartonella* (*Rochalimaea*) *henselae* causes cat-scratch disease, bacillary angiomatosis, peliosis hepatis, and fever in humans. *B. henselae* can be difficult to culture axenically, and as many as 5 weeks may be required before colonies are visible. We compared how different methods of blood collection and handling affect isolation of this pathogen. Blood specimens from *B. henselae*-infected cats were collected in both EDTA and Isolator blood-lysis tubes and were subsequently plated onto rabbit blood-brain heart infusion agar by using three different schedules: plating immediately, plating after 24 h at 25°C, and plating after 26 days at −65°C. Colonies were counted 14 and 35 days after plating. Blood collected in tubes containing EDTA, frozen at −65°C, and then plated on blood agar yielded a median of 60,000 CFU/ml, compared with 25,333 CFU/ml after collection in the Isolator tubes ($P < 0.01$). Frozen blood yielded the largest number of *B. henselae* colonies for any of the schedules tested. These results support previous observations that the Isolator system is more sensitive than tubes containing EDTA for isolation of *B. henselae* and suggest that, for cat blood, collection in tubes containing EDTA and subsequent freezing may further improve the sensitivity of detection of *B. henselae*.**

Bartonella (*Rochalimaea*) *henselae* is an important cause of illness in both immunocompromised and nonimmunocompromised humans; infection with this organism can lead to cat-scratch disease, bacillary angiomatosis, peliosis hepatis, and fever (1, 10, 22, 23, 28). However, the laboratory confirmation of a clinical diagnosis of these illnesses by isolation of *B. henselae* is infrequent. Several factors influence the potential to derive an isolate from a bacteremic patient, including the magnitude and duration of bacteremia, the laboratory's ability to culture the microbe, and the maintenance of organism viability from the time that the specimen is obtained from the patient to the time of cultivation.

Primary recovery of *B. henselae* often requires incubation for as long as 5 weeks, a CO_2-rich environment, and an enriched blood agar growth medium (18). The prolonged incubation precludes isolation as a method for rapid confirmation of a clinical diagnosis, increases the amount of incubator space required for routine cultures, and increases the chance of contamination. Liquid culture media and automated bacterial isolation systems have not been widely successful for the isolation of *B. henselae* (18, 29).

Collection of patient blood in both Isolator blood-lysis tubes (Wampole, Cranbury, N.J.) and tubes containing EDTA has been used to obtain human isolates of *B. henselae* (10, 18, 28, 29); however, to our knowledge, the sensitivities of these methods have not been experimentally compared. Isolator tubes contain sodium polyanetholsulfonate, an anticoagulant that inhibits the antibacterial effects of blood, and saponin, which lyses erythrocytes and leukocytes and which may serve to liberate intracellular organisms or those organisms grouped together on cell membranes (27). Blood cells collected in tubes containing EDTA are typically left unlysed.

Because of possible delays before being plated on growth media, blood specimens collected in a typical clinical setting may be frozen or may remain at room temperature for many hours. The Isolator system has been effective in isolating certain bacteria and fungi (4, 5, 7, 8). Processing can be done even after blood has remained at room temperature for as long as 16 h (27). However, the effects of temperature and time on the viability of *B. henselae* have not been evaluated, nor has recovery of *B. henselae* from EDTA-treated blood after different time and temperature treatments been investigated.

Cats support *B. henselae* bacteremias (9, 15, 19, 22, 26), and experimentally infected cats can provide a defined source of *B. henselae*-infected blood (17). We used infected cat blood to compare the sensitivities of the Isolator and EDTA blood collection methods for the isolation of *B. henselae*. We also compared the effects of plating the blood immediately, plating after 24 h at room temperature, and plating after prolonged freezing.

MATERIALS AND METHODS

Blood collection. Cats were experimentally infected with *B. henselae* Houston-1 as described elsewhere (18, 21). Blood specimens (1.0 ml) were collected with syringes and 22-gauge needles from five uninfected and five *B. henselae*-infected adult, laboratory-raised cats. A total of 1 ml of blood (0.5 ml each of infected and uninfected blood) was immediately transferred with clean needles into (i) a pediatric DuPont Isolator tube (Wampole) and (ii) a pediatric EDTA-containing tube (Becton Dickinson, Cockeysville, Md.). It was necessary to inoculate equal volumes of infected and uninfected blood so that there would be an adequate volume (i.e., 1 ml for each tube) to process the specimens under our protocol. All tubes were inverted three times to ensure adequate mixing of the blood.

Isolate recovery schedules. Feline blood specimens collected in Isolator tubes and tubes containing EDTA were processed according to three different schedules. One-third of each sample was inoculated onto rabbit blood heart infusion agar with rabbit blood (BBL Microbiology Systems [Becton Dickinson, Cockeysville, Md.]) within 2 to 6 h. Another aliquot was plated after 24 h at room temperature (25°C), and a third set was plated after freezing at −65°C for 26 days. The plates were used prior to the expiration dates suggested by the manufacturer.

Plating methods. Three 10-fold dilutions of variously treated *B. henselae*-infected cat blood or stock cultures of agar-grown *B. henselae* were made in brain heart infusion (BHI) broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.). A 200-μl pipette was used to inoculate 0.1 ml of each dilution onto the surface of rabbit blood agar plates (BBL Microbiology Systems [Becton Dickinson]). The plates were briefly held at a 45-degree angle to allow the

* Corresponding author. Mailing address: 2919 Galahad Dr. NE, Atlanta, GA 30345. Phone: (404) 329-8914. Fax: (404) 880-9305.

TABLE 1. Effects of temperature and method of blood collection for isolation of *B. henselae*

Day of colony counting	Time of plating	Freezing[a]	Median colony count (CFU/ml [range])		P value[b]
			EDTA-containing tubes (n = 5)	Isolator tubes (n = 5)	
14	Immediately	No	67 (20–400)	17,733 (4,000–30,667)	0.001
	24 h[c]	No	400 (67–1,200)	15,333 (4,000–31,333)	0.001
	26 days	Yes	61,333 (40,000–104,667)	22,000 (4,733–29,333)	0.012
35	Immediately	No	7,400 (100–12,867)	17,800 (1,000–30,667)	0.006
	24 h[c]	No	5,600 (400–10,667)	20,000 (3,933–35,000)	0.011
	26 days	Yes	60,000 (38,667–105,000)	25,333 (10,667–44,000)	0.019

[a] Frozen at −65°C for 26 days.
[b] P value of log (median colony count).
[c] Held for 24 h at 25°C before plating.

inocula to flow across the agar; mechanical spreading was not used. Titrations of blood samples were done in triplicate. All plates were placed in polyethylene bags to decrease contamination by saprophytic fungi and to help prevent desiccation of the agar and were incubated at 34 to 35°C in 5% CO_2. Plates were set agar side down initially and were then inverted after 3 to 4 days. All plates were examined for evidence of growth on day 14 and day 35 after plating, and colonies were counted at both times. The numbers of CFU per milliliter from cat blood samples were calculated from the plates at the terminal dilutions by using the following formula: CFU per milliliter = {(total CFU on all plates for each dilution/number of plates inoculated) × (dilution) × (0.1 ml inoculum plated) × [2 (1:1 infected blood to uninfected cat blood)]}. Medians and ranges were determined. P values were determined by performing the paired t test on the logarithm of the colony count.

Effect of repeated freezing. A single ampoule of agar-grown *B. henselae*, suspended in BHI broth, was repeatedly frozen to −85°C and thawed at room temperature. An aliquot of the suspension was removed after each freeze-thaw cycle and was titrated on rabbit blood agar plates to determine the numbers of viable CFU.

Effect of freezing the blood. To further investigate the mechanism of a change in colony counts after freezing, a *B. henselae* stock culture was added either to fresh unfrozen cat blood or to blood that had been frozen and thawed. *B. henselae* was immediately plated as described above.

RESULTS

The titer of *B. henselae* after repeated freeze-thaw cycles in BHI broth decreased in a roughly linear manner. A 50% reduction in titer was achieved after four freeze-thaw cycles (50% lethal dose, 4.03).

B. henselae colonies derived from infected cat blood collected in the Isolator tubes or after freezing with EDTA were generally more uniform in diameter and were more distinctly separated than those from blood collected in unfrozen EDTA-containing tubes. As expected, increasing dilutions of blood treated in Isolator tubes or frozen with EDTA showed a reciprocal relationship between increasing dilution and decreasing numbers of CFU per milliliter. However, unfrozen blood collected in EDTA-containing tubes did not regularly display a simple reciprocal relationship between the dilution and the numbers of CFU.

The median colony counts for Isolator tube and EDTA-treated blood samples are presented in Table 1. For blood plated immediately and after 24 h at 25°C, higher median colony counts were observed with the Isolator tubes at readings taken on days 14 and 35 after plating ($P < 0.01$). Median colony counts had significantly increased for the blood collected in EDTA-containing tubes by day 35, but were still less than counts for the blood collected in Isolator tubes ($P < 0.01$). Colony counts were slightly higher for blood from EDTA-containing tubes plated after 24 h at 25°C than for blood from EDTA-containing tubes plated immediately, but the difference was not significant.

In contrast, the highest colony counts for any temperature or blood collection system were obtained with blood plated after collection in tubes containing EDTA with subsequent freezing (Table 1). An increase in colony count was found in every individual specimen collected by this method (Fig. 1 and 2). Stock *B. henselae* colonies cultured with frozen-thawed cat blood showed mean colony counts of 6.8×10^7 CFU/ml, compared with mean colony counts of 1.9×10^7 CFU/ml for unfrozen cat blood ($P = 0.02$) (data not shown).

Colonies derived from unfrozen blood collected in EDTA-containing tubes were more heterogeneous in size than those derived either from frozen samples or from Isolator tube samples. Previous experiments have shown that heterogeneous colonies observed with blood (unfrozen) from tubes containing EDTA are genotypically identical, as judged by PCR-restriction endonuclease fragment length polymorphism analysis (16). When large and small colonies were subcultured, they yielded similarly sized, uniform colonies (data not shown).

Colony counts did not differ significantly when agar-grown, aggregate-containing *B. henselae* colonies were diluted with BHI broth compared to those when the colonies were diluted with Isolator lysis solution (data not shown).

DISCUSSION

The findings of the present study suggest that cell lysis, disruption of cell membranes, and/or dispersal of aggregates of bacteria improve the sensitivity and rapidity of colony formation of *B. henselae*. By light microscopy, complete cell lysis was seen with blood from both the Isolator tubes and tubes containing EDTA with subsequent freezing.

The lack of significant differences between colony counts when agar-grown, aggregate-containing *B. henselae* colonies

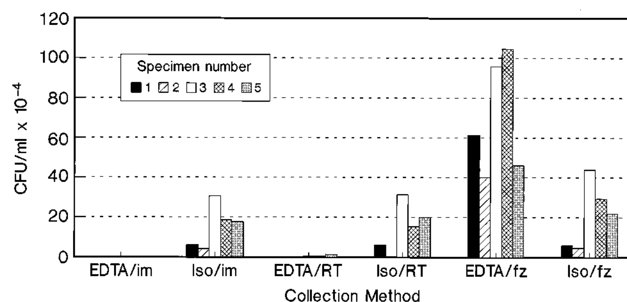

FIG. 1. Mean colony counts for EDTA-containing tubes versus those for Isolator tubes on day 14. im, immediate plating; RT, plating after storage at room temperature; fz, plated after freezing.

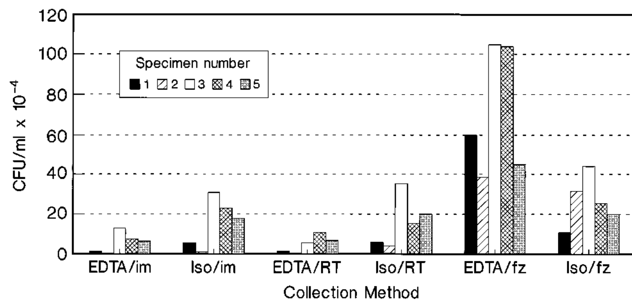

FIG. 2. Mean colony counts for EDTA-containing tubes versus those for Isolator tubes on day 35. See legend to Fig. 1 for definitions of abbreviations.

were diluted with Isolator tube lysis solution and those when they were diluted with BHI broth suggests that dissolution of bacterial aggregates by the Isolator tube solution is probably not the major factor responsible for increasing the plating sensitivity of blood-borne *B. henselae*. This experiment also demonstrates that there is no immediate negative effect of the Isolator tube solution on the viability of *B. henselae.*

Our results suggest that host cell lysis improves *B. henselae* recovery and are consistent with previous findings that at least some of the *B. henselae* organisms involved in feline bacteremias may be intracellular (11). Persistent bacteremia and relapses despite the use of antibiotics in humans (12, 18), electron microscopy studies of *B. henselae* in cats (11) and in vitro (2), enhanced polymorphonuclear leukocyte phagocytosis in the presence of *B. henselae* (24), and the need to treat humans with *B. henselae* infection with antibiotics that have good intracellular penetration (12, 18, 25) also support this theory. In addition, there is evidence that other *Bartonella* species, such as *B. bacilliformis*, are intracellular (3, 6, 13, 14) and reside within erythrocytes. Electron microscopy studies have suggested that *B. henselae* may also be intraerythrocytic (11).

If individual colonies are produced by each viable *B. henselae* organism present outside a host cell and by each host cell infected with one or more *B. henselae* organisms (regardless of the number of *B. henselae* organisms in the cell), then the dramatically higher colony counts after freezing-lysis (e.g., increasing from 128 to 69,600 CFU/ml) imply that a few host cells may be infected with a large mean number of *B. henselae* organisms.

This hypothesis appears to be consistent with the observed heterogeneous colony size associated with *B. henselae* colonies in fresh EDTA-treated blood; larger colonies may represent single host cells bearing large bacterial loads. Conversely, after cell disruption, colony morphology (derived from single organisms) would be expected to be uniform, as observed. Autoagglutination of cultured *B. henselae* cells has been noted in previous studies and could also lead to large colonies (18, 20).

Once treated either with Isolator tube solution or by freezing in EDTA, colony counts did not significantly increase when incubation was extended from 2 to 5 weeks, suggesting that host cell disruption is a critical factor in the timing of colony formation. Inactivation of a growth inhibitor by freezing-thawing may also contribute to the findings; our preliminary findings from adding frozen-thawed blood to a stock *B. henselae* culture suggest that an inhibitor may account for a small portion of the increase in colony counts after freezing.

Practical clinical considerations for determining the method of choice for the isolation of *B. henselae* include the relative initial cost of Isolator tubes and EDTA-containing tubes, the number and practicality of steps required to handle the various specimen types, and the length of time needed to process specimens. Isolator tubes have a considerably higher unit cost than collection tubes containing EDTA, and tubes containing EDTA are more commonly found in many health care environments. Pediatric Isolator tubes do not require increased processing time; however, the larger adult-size Isolator tubes undergo centrifugation (and subsequent resuspension) to concentrate and recover the bacterial organisms in the pelleted material (27). Frozen blood in tubes containing EDTA provided the most sensitive rate of recovery of any of the methods that we tested. Although we froze the blood for 26 days, a subsequent experiment showed no significant differences in mean colony counts when blood was frozen for 1 day compared with that when blood was frozen for 30 days ($P = 0.79$). However, freezing of blood in tubes containing EDTA requires that the blood be steriley transferred from the glass collection tube to an appropriate shatterproof vessel prior to freezing; this may be regarded as an extra step, prone to contamination, and is potentially hazardous.

This study suggests that *B. henselae* in blood samples is relatively stable at room temperature for 24 h (i.e., enough time to transport a specimen to a reference laboratory). Similarly, it is clear that viability, if anything, is enhanced by a single frozen storage of blood specimens.

These culture results were derived with blood from cats infected with *B. henselae*. Anecdotal accounts of resistance to freezing have previously been noted for *B. henselae* recovered from human blood (18). We assume that the basic properties of resistance to freezing and stability at room temperature would also apply to the *B. henselae* bacteremia of humans. *B. henselae* bacteremia in humans appears to be primarily, but not exclusively (28), a phenomenon associated with immunosuppression (18). If cell lysis does prove to be as important to the recovery of *B. henselae* from human samples as it is to the recovery of the organism from infected cats, it would further suggest that there is an intracellular component to the human infection.

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