Rapid Susceptibility Testing of Lyme Disease Spirochetes by Flow Cytometry

Dean A. Jobe, MS*; Steven M. Callister, PhD*†; Steven D. Lovrich, PhD*; and Ronald F. Schell, PhD‡,§,¶

ABSTRACT

Flow cytometry has recently become an effective tool for rapidly determining antimicrobial susceptibilities of microorganisms pathogenic to humans. In this study, we developed an in vitro assay that used flow cytometry to detect rapidly (18 hours) the minimum bactericidal concentrations (MBC) of antimicrobial agents against several isolates of *Borrelia burgdorferi* sensu lato. Acridine orange fluorescence intensity and number of events were used to detect killed spirochetes incubated in Barbour-Stoenner-Kelly (BSK) medium containing decreasing concentrations of antimicrobial agents. The flow cytometric susceptibility assay accurately predicted MBC values for amoxicillin (0.06 µg/mL), cefotaxime (0.06 µg/mL), ceftriaxone (0.03 µg/mL), doxycycline (0.25 µg/mL), and erythromycin (0.13 µg/mL). In addition, the flow cytometric procedure rapidly detected significant variations in MBC values among *Borrelia* isolates. We conclude that flow cytometry is a rapid and accurate method for determining MBC values of antimicrobial agents against *B burgdorferi* sensu lato. Additionally, the use of flow cytometry will aid in the rapid evaluation of newly developed antimicrobial agents and provide a more accurate assessment of in vivo concentrations necessary to eliminate *B burgdorferi* sensu lato infections.

Key words: *Borrelia burgdorferi*, flow cytometry, susceptibility testing

INTRODUCTION

Lyme borreliosis is the most common tick-associated illness in the world.[1] This illness is acquired by humans through the bite of *Ixodes* species ticks[2] infected with *Borrelia burgdorferi* sensu lato (Bb) (*Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*). The clinical manifestations of early Lyme borreliosis, including constitutional symptoms such as fatigue, headache, mild stiff neck, arthralgias, myalgias, and fever, are often accompanied by a skin rash, erythema migrans.[3,4] Persistence of Bb can lead to more severe clinical manifestations including secondary annular lesions, meningitis, Bell's palsy, radiculoneuritis, and atrioventricular heart block.[4-6] Furthermore, chronic arthritis may develop weeks to months after infection.[4]

The variability and occasionally protracted nature of Lyme borreliosis makes it difficult to assess the effectiveness of antimicrobial therapy. Optimal treatment regimens, particularly for patients with late-stage or persistent disease, are strongly debated because little is known about the pharmacodynamic interaction between the antimicrobial agent and Bb. In addition, the slow growth rate and fastidious nature of Bb organisms has hindered many investigations by delaying susceptibility testing by conventional methods. Furthermore, it is difficult to accurately determine the viability of Bb by darkfield microscopy, especially when spirochetes become clumped or exhibit impaired motility. In addition, interpretation of growth based on color change can be difficult. For instance, contaminating organisms can cause color changes in Barbour-Stoenner-Kelly (BSK) medium, which would interfere with interpretation of endpoints.

*From the *Microbiology Research Laboratory and †Section of Infectious Diseases, Gundersen/Lutheran Medical Center, La Crosse, Wisconsin; ‡Wisconsin State Laboratory of Hygiene, §Departments of Medical Microbiology and Immunology and ¶Bacteriology, University of Wisconsin, Madison, Wisconsin.*

Address correspondence to Steven M. Callister, PhD, Microbiology Research Laboratory, Gundersen/Lutheran Medical Center, 1836 South Avenue, La Crosse, WI 54601.

Oxidation of BSK medium can also kill Bb organisms and produce falsely lowered susceptibility values. Collectively, these factors make conventional methods labor-intensive, time consuming, and difficult to interpret.

Recently, we showed that susceptibility testing of other slow-growing microorganisms such as *Mycobacterium tuberculosis*,[7,8] nontuberculosis mycobacteria,[9] and *Candida albicans*[10] could be rapidly accomplished by using flow cytometry. Results of tests were available in 24 hours or less. In this report, we show that flow cytometry can be used to detect rapidly (18 hours) the minimum bactericidal concentrations (MBC) of antimicrobial agents against Bb.

MATERIALS AND METHODS

Antimicrobial Agents

Amoxicillin and erythromycin (Sigma Chemical Co., St. Louis, MO); cefotaxime (Hoechst-Roussel Pharmaceuticals, Sommerville, NJ); ceftriaxone (Roche Laboratories, Belvidere, NJ); and doxycycline (Pfizer, Inc., Groton, CT) were obtained as standard powders and prepared according to the manufacturer or distributor recommendations. Stock solutions contained 3200 μg of each antimicrobial agent per milliliter of sterile distilled water.

Organisms

Bb isolates 297 (human spinal fluid, Connecticut); B31 (*Ixodes scapularis*, New York); *B garinii* isolates LV4 and PBi (human spinal fluid, Europe); *B afzelii* isolates J1 (*Ixodes persulcatus*, Japan); and BV1 (human blood, Europe) were cultured for 72 hours at 32°C in BSK medium to a concentration of 5×10^7 spirochetes per milliliter. Then, 500 μL samples were dispensed into 1.5 mL sterile vials (Sarstedt Inc., Newton, NC), sealed, and stored at -70°C until used. When needed, a frozen suspension of spirochetes was thawed and used to inoculate fresh BSK. Spirochetes were enumerated using a Petroff-Hausser counting chamber.

Susceptibility Assays

Minimum bactericidal concentration values for 5 antimicrobial agents against isolates of Bb were determined by using a conventional macrodilution technique[11,12] and flow cytometry. Briefly, freshly-prepared stock solutions of each antimicrobial agent were serially diluted (16 to 0.008 μg/mL) in fresh BSK medium. Log phase (72 hours) cultures of Bb isolates were diluted with fresh BSK to a final concentration of 10^6 spirochetes per milliliter. For macrodilution assays, 2 mL of each Bb suspension were combined with 2 mL of each dilution of antimicrobial agent in sterile 13 × 100 mm polystyrene culture tubes (Becton-Dickinson, Lincoln Park, NJ) and incubated at 32°C for 7 days. For flow cytometric assays, 100 μL of each Bb suspension was combined with 100 μL of each dilution of antimicrobial agent in sterile 1.5 mL microcentrifuge tubes (Sarstedt) and incubated at 32°C for 18 hours. Minimum inhibitory concentration values against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were determined using NCCLS guidelines to ensure appropriate activity of each antimicrobial agent.[13]

Determination of Minimum Bactericidal Concentrations

Macrodilution. Suspensions of antimicrobial agents in which viable spirochetes could not be detected by using darkfield microscopy were subcultured (10% v/v) into 6 mL of BSK containing no antimicrobial agents and incubated for 7 days. The lowest concentration in which viable spirochetes could not be detected by using darkfield microscopy was considered the MBC. Nonmotile Bb organisms were considered nonviable.[14] All assays were performed in duplicate.

Flow cytometry. Following 18 hours of incubation, 100 μL of each assay suspension was transfered into 12 × 75 mm polystyrene culture tubes (Fisher Scientific, Chicago, IL) containing 400 μL of 0.20 μm filter-sterilized phosphate buffered saline (PBS; 0.01 mol/L, pH 7.2) and 50 μL of acridine orange (AO) (5.4×10^{-9} mol/L). Suspensions were gently vortexed and data were acquired using a FACScan single laser flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Initially, viable Bb organisms were detected and differentiated from BSK particles by using side angle light scatter and AO fluorescence intensity parameters. Live gating was performed on dot plots of Bb organisms during data acquisition to exclude debris. Events were acquired for 60 to 90 seconds in the list mode. Fluorescence histograms for each sample were analyzed by using FACScan LYSYS II software. Markers were established for viable Bb organisms based on their binding of AO. The intensity of AO fluorescence and the number of Bb (events) were used to detect bactericidal activity.

Growth Assays

Following flow cytometric analysis, Bb organisms contained in 100 μL of each assay suspension were collected by using 0.2 μm microcentrifuge filter units (Corning Costar Corp., Cambridge, MA) spun at 1500 rpm for 3 minutes. The filters were then washed with 500 μL of sterile PBS to remove residual antimicrobial agents. Following washing, the filters containing spirochetes were resuspended in 500 μL of fresh BSK medium and serially diluted (10-fold) to enumerate survivors. All cultures

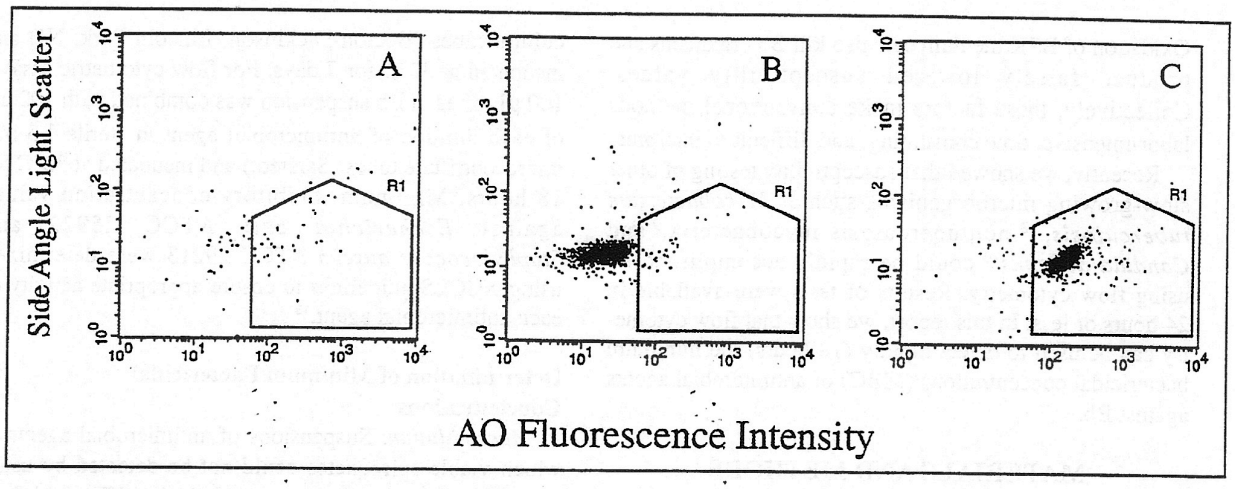

Figure 1. Intensity of acridine orange (AO) fluorescence versus side angle light scatter of B burgdorferi *in the absence (B) or presence (C) of AO in BSK medium. Barbour-Stoenner-Kelly medium without* B burgdorferi *(A) was included as a control.*

were incubated for 14 days and examined periodically for the presence of motile spirochetes by using dark-field microscopy.

RESULTS

Detection of Bb by Flow Cytometry

Viable Bb organisms (Figure 1B) were easily detected and differentiated from particles in BSK medium (Figure 1A) by monitoring side angle light scatter and AO fluorescence intensity parameters. Few background particles were detected in BSK medium (Figure 1A). When Bb organisms were exposed to AO (Figure 1C), they were readily detected and differentiated from Bb organisms not exposed to AO (Figure 1B). When these experiments were repeated with other Bb isolates, similar results were obtained.

Establishment of Gates (regions) for Detection of Viable and Killed Bb

Subsequently, viable organisms were incubated in BSK containing 0.03 µg of ceftriaxone per milliliter. The mean channel of AO fluorescence intensity (MCF) for the ceftriaxone-treated spirochetes increased significantly (Figure 2B) compared with the MCF of AO fluorescence intensity of the drug-free control (Figure 2A). In addition, the number of Bb (events) in the sample treated with ceftriaxone (Figure 2D) was significantly less than the number of spirochetes in the drug-free control (Figure 2C). Similar results were obtained using 0.06 µg of amoxicillin per milliliter, 0.06 µg of cefotaxime per milliliter, 0.25 µg of doxycycline per milliliter, and 0.13 µg of erythromycin per milliliter. When isolates of *B burgdorferi*, *B garinii*, and *B afzelii* were exposed to these antimicrobial agents, uptake of AO by the drug-treated isolates of Bb was significantly more than the uptake of AO by the drug-free controls.

Determination of the Susceptibility of Bb to Antimicrobial Agents by Flow Cytometry

The effects of various concentrations of amoxicillin, cefotaxime, ceftriaxone, doxycycline, and erythromycin were determined based on the intensity of AO fluorescence and the number of Bb (events) after exposure to these antimicrobial agents for 18 hours. In general, the intensity of AO fluorescence increased rapidly when Bb were exposed to increasing concentrations of the antimicrobial agents, while the number of Bb (events) rapidly decreased (Figure 3 A-E). When the number of Bb (events) in samples decreased to approximately 10^3, viable spirochetes were not recovered by subculture to fresh BSK medium. The point at which the AO fluorescence intensity and events curves intersected approximated the MBC of each antimicrobial agent for Bb. These MBC values were 0.06 µg/mL for amoxicillin, 0.06 µg/mL for cefotaxime, 0.03 µg/mL for ceftriaxone, 0.25 µg/mL for doxycycline, and 0.13 µg/mL for erythromycin. The MBC's were confirmed by subculturing washed *B burgdorferi* 297 collected on filters. Results of subculturing experiments confirmed that *B burgdorferi* 297 organisms were killed (data not shown). These values correlated with conventional macrodilution MBCs, with the exception of amoxicillin. The conventional macrodilution MBC for amoxicillin was 16 µg/mL, while the flow cytometric MBC was only 0.06 µg/mL (Table 1). This was likely because of the breakdown of amoxicillin during the long incubation period required for the macrodilution method. Other investigators have shown significant

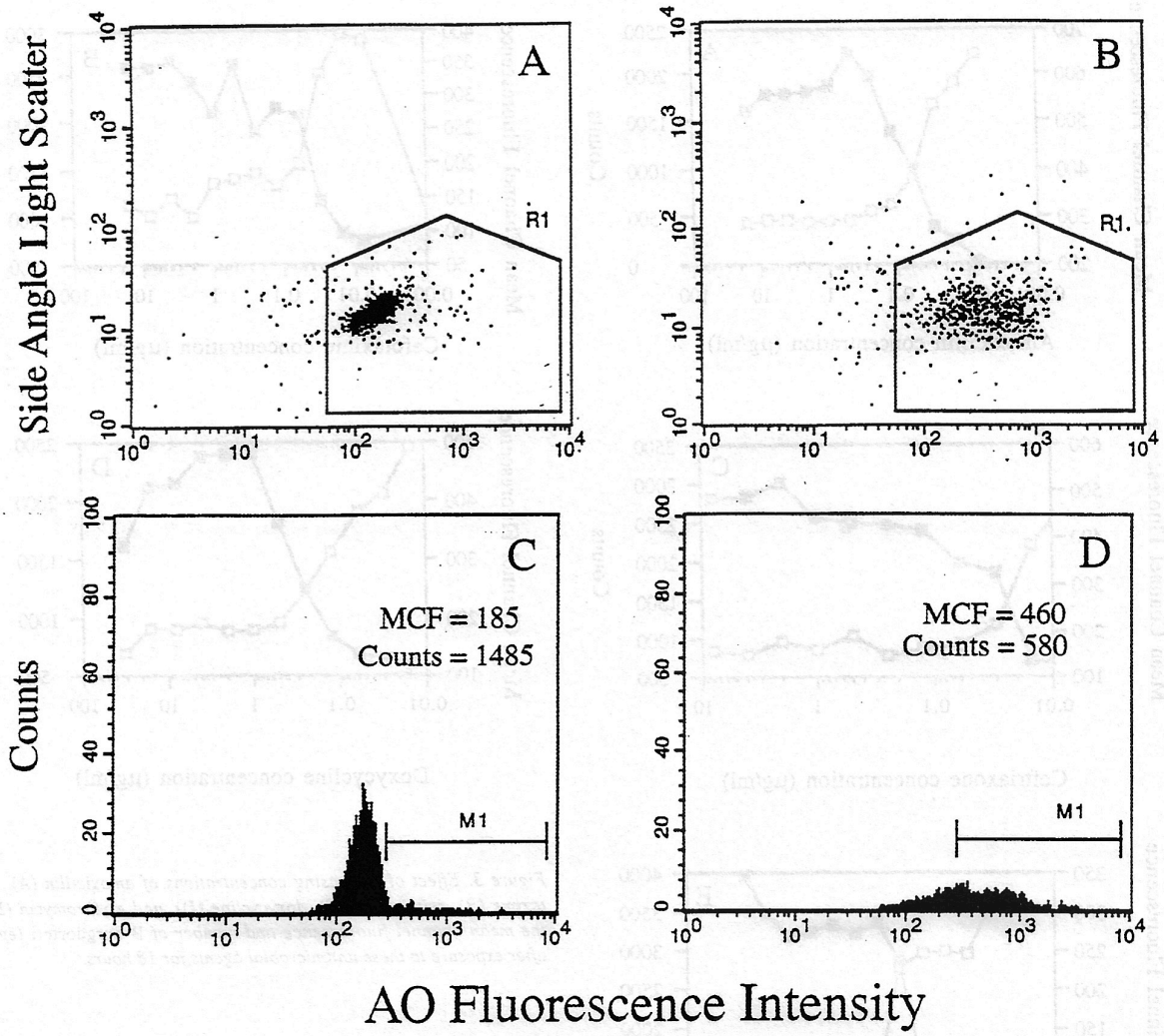

Figure 2. Mean channel fluorescence (acridine orange (AO) fluorescence intensity) versus side angle light scatter of B burgdorferi *in the absence (A) and presence (B) of 0.03 µg/mL ceftriaxone. The lower figures are histogram profiles of the dot plots (A and B).*

Table 1. Comparison of MBC values[a] for five antimicrobial agents against B burgdorferi *sensu stricto 297 obtained by using flow cytometry and broth macrodilution methods.*

Antimicrobial	Minimum borreliacidal concentration[b] (µg/mL)	
	Flow cytometry	Macrodilution
Amoxicillin	0.06	16
Cefotaxime	0.06	0.06
Ceftriaxone	0.03	0.04
Doxycycline	0.25	0.50
Erythromycin	0.13	0.06

[a] *Similar results were obtained when other B burgdorferi sensu lato isolates were tested.*

[b] *Geometric mean of duplicate samples.*

loss (>25%) of activity of doxycycline[21] and ceftriaxone[20,21] after 24 to 72 hours of incubation at 37°C.

Based on these results, an operational definition of susceptibility was established to distinguish changes in intensity of AO fluorescence and numbers of Bb (events), which would accurately predict the MBCs. If the intensity of AO fluorescence of the Bb culture containing antimicrobial agents was 40% more than the intensity of AO fluorescence obtained with the drug-free culture, the MBC was identified. Likewise, a 50% or more decrease in the number of Bb (events) also predicted the MBC. When these flow cytometric definitions were applied to five other isolates of Bb tested with these antimicrobial agents, the MBCs were identified (Table 2). The MBC values

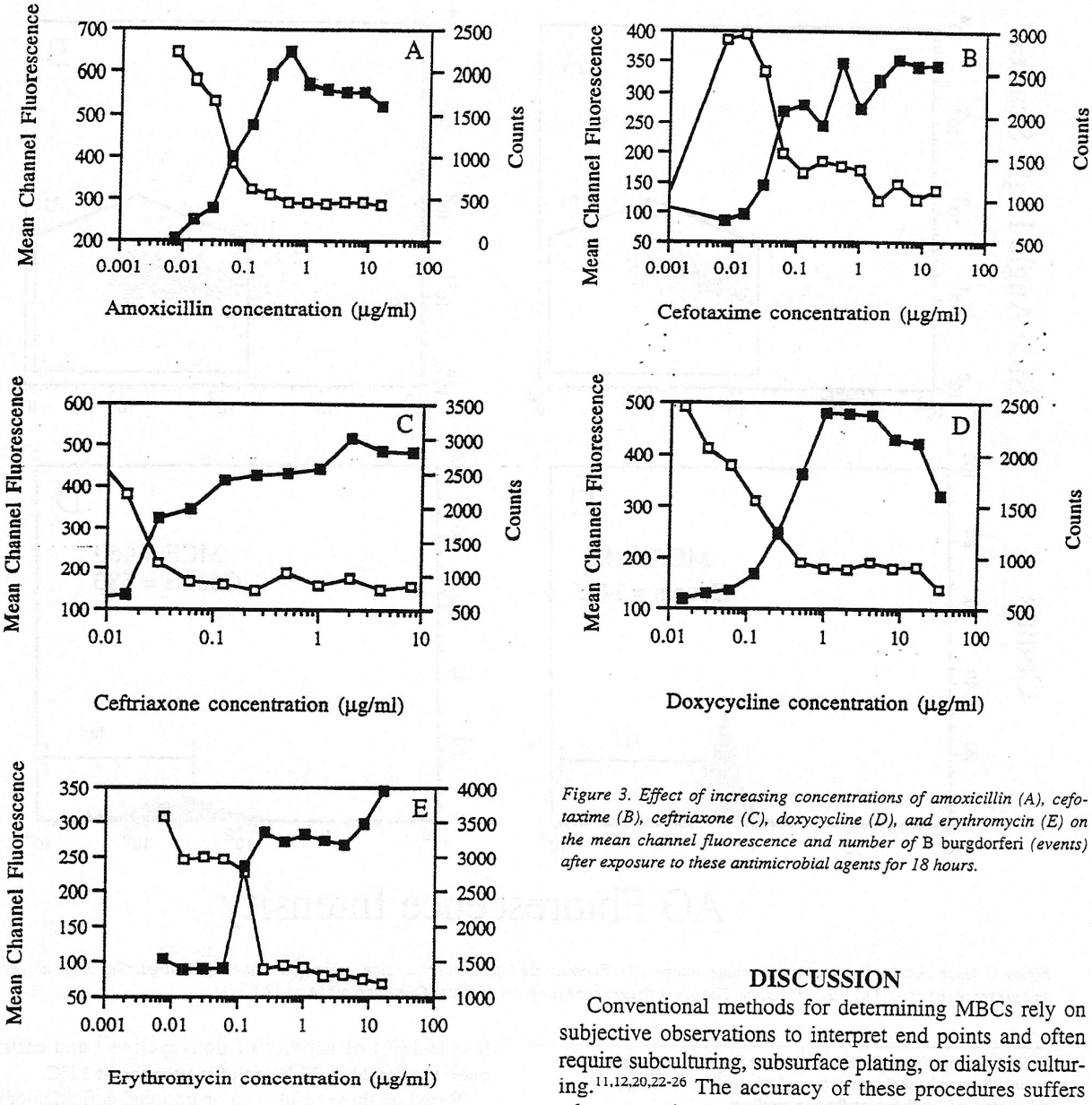

Figure 3. Effect of increasing concentrations of amoxicillin (A), cefotaxime (B), ceftriaxone (C), doxycycline (D), and erythromycin (E) on the mean channel fluorescence and number of B burgdorferi *(events) after exposure to these antimicrobial agents for 18 hours.*

obtained for each isolate were similar and corresponded closely with previously published results using conventional susceptibility testing methods.[11,12,20,22-25] However, the flow cytometric procedure detected some significant differences in MBC values among different Bb isolates. For example, the MBC values of doxycycline against *B burgdorferi* B-31 was 0.13 µg/mL. In contrast, *B garinii* LV4 and *B afzelii* J1 had doxycycline MBC values of 4.0 µg/mL.

DISCUSSION

Conventional methods for determining MBCs rely on subjective observations to interpret end points and often require subculturing, subsurface plating, or dialysis culturing.[11,12,20,22-26] The accuracy of these procedures suffers when organisms replicate slowly because of increased incubation times and the instability of antimicrobial agents. A flow cytometer is capable of simultaneously collecting quantitative and qualitative data by detecting individual cells. This allows for rapid and precise identification and characterization of cells contained within a heterogenous population. In addition, the viability of microorganisms contained in a population can be determined by monitoring the uptake of fluorescent dyes.[7-10,15-19] These characteristics of flow cytometry have led investigators to explore its use as an alternative approach to conventional susceptibility testing methods. Results of these studies

Table 2. Flow cytometric MBC values for five antimicrobial agents against B burgdorferi *sensu stricto,* B garinii, *and* B afzelii *isolates.*

Antimicrobial	Minimum Borreliacidal Concentration* (μg/mL)				
	CFT	CTX	DOX	AMX	ERY
B burgdorferi ss					
B31	0.04	0.06	0.13	0.13	0.13
B garinii					
LV4	0.02	0.06	4	1	1
PBi	0.04	0.13	0.50	0.06	0.02
B afzelii					
BV1	0.08	0.13	0.50	2	0.06
J1	0.02	0.06	4	2	0.03

**Geometric mean of duplicate samples.*
Abbreviations: CFT = Ceftriaxone; CTX = Cefotaxime; DOX = Doxycycline; AMX = Amoxicillin; ERY = Erythromycin.

have demonstrated that flow cytometry is useful for determining the antimicrobial susceptibilities of slow-growing bacterial and fungal pathogens.[7-10,15-18]

In this investigation, we developed a flow cytometric procedure to determine MBCs of Bb more rapidly. The ability to obtain susceptibility results in 18 hours is a significant improvement over the 13 to 15 days required by conventional Bb susceptibility assays.[11,12,20] By monitoring intensity of AO fluorescence and the number of Bb (events), flow cytometry could easily discriminate between living and nonviable Bb. Using a 40% increase in AO fluorescence intensity or a 50% decrease in the number of Bb (events) to predict MBCs appeared accurate. When concentrations of antimicrobial agents were below the MBC values, AO fluorescence intensity and the number of Bb (events) remained nearly identical to those values obtained with spirochetes incubated in drug-free BSK medium. Although testing of more isolates of Bb might fine tune the determination of cut-off values, these values are unlikely to differ significantly from our selected values.

In addition, the accuracy of susceptibility testing of Bb may improve by performing the flow cytometric assay. We detected significant variations in the MBCs of antimicrobial agents among several Bb isolates. The ability of flow cytometry to rapidly determine MBC concentrations of antimicrobial agents against Bb should improve the correlation between in vitro susceptibility results and clinical efficacy. This will be especially important when evaluating antimicrobial agents that are unstable in solution or have been newly developed as potential therapies for Lyme borreliosis. In addition, the ease and objectivity of the flow cytometric procedure makes it suitable for determining MBCs against large numbers of Bb isolates.

In conclusion, we demonstrated that susceptibility testing of Bb could be accomplished by using flow cytometry to monitor the uptake of AO and enumerate viable and killed Bb organisms in drug-free and antimicrobial agent-containing medium. Most importantly, the flow cytometric susceptibility test was rapid, reproducible, and simple to perform.

ACKNOWLEDGMENT

This work was supported by the Gundersen Lutheran Medical Foundation.

REFERENCES

1. Centers for Disease Control. Lyme disease. Morbid Mortal Weekly Report 1997;46:531-535.

2. Barbour AG, Tessier SL, Todd WJ. Lyme disease spirochetes and ixodid ticks share a common surface antigen defined by monoclonal antibody. Infect Immun 1983;41:795-804.

3. Steere AC, Bartenhagen NH, Craft JE, Hutchinson GJ, Neuman JH, Rahn DW, Sigal LH, Spieler PN, Stern KS, Malawista SE. The early clinical manifestations of Lyme disease. Ann Intern Med 1983;99:76-82.

4. Steere AC, Schoen RT, Taylor E. The clinical evolution of Lyme arthritis. Ann Intern Med 1987;107:725-731.

5. Pachner AR, Steere AC. The triad of neurologic manifestations of Lyme disease: meningitis, cranial neuritis, and radiculoneuritis. Neurol 1985;35:47-53.

6. Steere AC, Batsford WP, Weinberg AM, Alexander J, Berger HJ, Wolfson S, Malawista SE. Lyme carditis: cardiac abnormalities of Lyme disease. Ann Intern Med 1980;93:8-16.

7. Norden MA, Kurzynski TA, Bownds SE, Callister SM, Schell RF. Rapid susceptibility testing of *Mycobacterium tuberculosis* (H37Ra) by flow cytometry. J Clin Microbiol 1995;33:1231-1237.

8. Kirk SM, Schell RF, Moore AV, Callister SM, Mazurek GH. Flow cytometric testing of susceptibilities of *Mycobacterium tuberculosis* isolates to ethambutol, isoniazid, and rifampin. J Clin Microbiol 1998;36:1568-1573.

9. Bownds SE, Kurzynski TA, Norden MA, Dufek JL, Schell RF. Rapid susceptibility testing for nontuberculosis mycobacteria using flow cytometry. J Clin Microbiol 1996;34:1386-1390.

10. Kirk SM, Callister SM, Lim LCL, Schell RF. Rapid susceptibility testing of *Candida albicans* by flow cytometry. J Clin Microbiol 1997;35:358-363.

11. Agger WA, Callister SM, Jobe DA. In vitro susceptibilities of *Borrelia burgdorferi* to five oral cephalosporins and ceftriaxone. Antimicrob Agents Chemother 1992;36:1788-1790.

12. Johnson RC, Kodner C, Russell M. In vitro and in vivo susceptibility of the Lyme disease spirochete, *Borrelia burgdorferi*, to four antimicrobial agents. Antimicrob Agents Chemother 1987;31:164-167.

13. National Committee for Clinical Laboratory Standards. 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 2nd ed. M7-A2. National Committee for Clinical Laboratory Standards, Villanova, PA.

14. Callister SM, Schell RF, Lovrich SD. Lyme disease which detects killed *Borrelia burgdorferi*. J Clin Microbiol 1991;29:1773-1776.

15. Ordonez JR, Wehman NM. Rapid flow cytometric antibiotic susceptibility assay for *Staphylococcus aureus*. Cytometry 1993;14:811-818.

16. Roth BL, Poot M, Yue ST, Millard PJ. Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. Appl Environ Microbiol 1997;63:2421-2431.

17. Green L, Petersen B, Steimel L, Haeber P, Current, W. Rapid determination of antifungal activity by flow cytometry. J Clin Microbiol 1994;32:1088-1091.

18. Ramani R, Ramani A, Wong SJ. Rapid flow cytometric susceptibility testing of *Candida albicans*. J Clin Microbiol 1997;35:2320-2324.

19. Lebaron P, Catala P, Parthuisot N. Effectiveness of SYTOX green stain for bacterial viability assessment. Appl Environ Microbiol 1998;64:2697-2700.

20. Dever LL, Jorgensen JH, Barbour AG. In vitro antimicrobial susceptibility testing of *Borrelia burgdorferi*: a microdilution method and time kill studies. J Clin Microbiol 1992;30:2692-2697.

21. Kersten A, Poitschek C, Rauch S, Aberer E. Effects of penicillin, ceftriaxone, and doxycycline on morphology of *Borrelia burgdoferi*. Antimicrob Agents Chemother 1995;39:1127-1133.

22. Johnson RC, Kodner CB, Jurkovich PJ, Collins JJ. Comparative in vitro and in vivo susceptibilities of the Lyme disease spirochete, *Borrelia burgdorferi*, to cefuroxime and other antimicrobial agents. Antimicrob Agents Chemother 1990;34:2133-2136.

23. Johnson SE, Klein GC, Schmid GP, Feeley JC. Susceptibility of the Lyme disease spirochete to seven antimicrobial agents. Yale J Biol Med 1984;57:549-553.

24. Levin JM, Nelson JA, Segreti J, Harrison B, Benson CA, Strle F. In vitro susceptibility of *Borrelia burgdorferi* to 11 antimicrobial agents. Antimicrob Agents Chemother 1993;37:1444-1446.

25.Mursic VP, Wilske B, Schierz G, Holmburger M, SuB E. In vitro and in vivo susceptibility of *Borrelia burgdorferi*. Europ J Clin Microbiol 1987;6:424-426.

26. Stierstedt SH, Wretlind B. Dialysis culture enables more accurate determination of MIC of benzylpenicillin for *Borrelia burgdorferi* than does conventional procedure. Antimicrob Agents Chemother 1996;40:2882-2883.