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Source: Journal of Wildlife Diseases, 31(3) : 345-351

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-31.3.345

Journal of Wildlife Diseases, 31(3), 1995, pp. 345–351

EAR BIOPSY LOCATION INFLUENCES DETECTION OF *BORRELIA BURGDORFERI* BY PCR, BUT NOT BY CULTURE IN NATURALLY INFECTED *PEROMYSCUS LEUCOPUS*

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ABSTRACT: We determined if the ear biopsy location affected detection of *Borrelia burgdorferi* when either culture or the polymerase chain reaction (PCR) was used among 50 white-footed mice (*Peromyscus leucopus*), live-captured in a Lyme disease enzootic area in Maryland (USA) between March and October of 1991 and 1992. The infection status of individual mice was determined by organ culture; ear biopsy samples were obtained from the peripheral and central part of the ear for detection of *B. burgdorferi* by culture and by PCR. Overall, *B. burgdorferi* was cultured from one or more tissue samples in 33 (66%) of 50 captured mice. Among infected mice, *B. burgdorferi* was detected by culture in 29 (88%) of 33 peripheral and 28 (85%) of 33 central ear biopsy samples. By PCR it was detected in 24 (73%) of 33 peripheral and all 33 central samples ($P = 0.002$). Detection of *B. burgdorferi* by culture was independent of the ear biopsy location; however, the organism was detected by PCR with greater frequency in central ear biopsy samples as compared to peripheral samples. Agreement between culture and PCR was moderate (Kappa = 0.64) on peripheral ear samples and excellent (Kappa = 0.79) on central samples. We propose that when ear biopsy samples are used to detect *B. burgdorferi* by PCR in wild-caught *P. leucopus*, removal of biopsy samples from the central part of the ear will achieve maximum sensitivity and will achieve the highest concordance between assays when both culture and PCR of ear biopsy samples are conducted in parallel.

Key words: *Borrelia burgdorferi*, *Peromyscus leucopus*, Lyme disease, zoonotic disease.

INTRODUCTION

Longitudinal studies of zoonotic disease agents in free-living reservoir animals require methods for obtaining repeated specimens of tissue and blood from individual animals. These methods should permit a sensitive and specific determination of infection by the pathogen of interest and carry a low risk of causing morbidity or mortality in the animal. The ear biopsy method is an efficient technique for obtaining tissue samples from naturally and experimentally infected rodents for detecting *Borrelia burgdorferi*, the causative agent of Lyme disease (Sinsky and Piesman, 1989; Hofmeister et al., 1992).

The optimal methods of obtaining ear tissue samples from wild-caught *Peromyscus leucopus*, the primary mammalian reservoir of *B. burgdorferi* (Mather et al., 1989), have not yet been fully explored. In sampling the ears of two hamsters experimentally infected with *B. burgdorferi*, Sinsky and Piesman (1989) observed that ear biopsy samples from the peripheral part of the ear were more likely to be culture-negative, or to produce less dense growth of spirochetes, than samples removed from the central part of the ear; we propose that the sensitivity of the ear biopsy technique may depend on sampling location. In captured *P. leucopus*, we have observed that the peripheral margins and the bases of ears are the primary sites of parasitism by feeding *Ixodes scapularis* ticks, whereas the central part of the ear is parasitized less frequently. In addition, the sampling location also may affect the agreement between culture and the polymerase chain reaction (PCR) on detection of *B. burgdorferi* in those studies in which both detection methods are used. The agreement

between these detection methods was found to be excellent on ear biopsy tissue (Hofmeister et al., 1992); however, the role of sampling location on the agreement between methods was not examined. Hence, the optimal location on the ear for biopsy remains unclear.

Our objectives were to determine if sampling the peripheral or central portion of ear affected the ability of detect *B. burgdorferi* in naturally infected *P. leucopus*, and to determine if culture and PCR were equally reliable in detecting the organism at either site in wild-caught mice.

MATERIALS AND METHODS

Fifty sub-adult or adult *P. leucopus* were live-captured in Sherman traps (H. B. Sherman Traps Inc., Tallahassee, Florida, USA) between March and October, 1991 and 1992, in a deciduous forest located within a Lyme disease enzootic area of Baltimore County, Maryland (USA). Captured mice were transported to the laboratory where they were euthanized with CO_2 and sampled in a biological safety cabinet. Single kidney, spleen, and urinary bladder samples, consisting of approximately one third of each organ, were aseptically removed from each mouse and separately placed directly in Barbour-Stoenner-Kelly II (BSK) culture media (Barbour, 1984) containing 10 μg of rifampin (Sigma Chemical Company, St. Louis, Missouri, USA), 4 μg of amphotericin B (Sigma), and 1,000 μg of phosphomycin (Sigma) per ml. From each mouse, in addition to organ tissues, two ear biopsy samples were removed from the peripheral part; the removed tissue included the outer margin of the ear. Two additional samples from the same ear were removed for culture from the central part; the removed tissue was approximately equidistant from the outer margins of the ear. Paired ear biopsy samples were removed each location because a small percentage (<10%) of ear cultures were expected to be contaminated with adventitious organisms. After cleaning the outside surface of the ear with a cotton swab soaked in 95% ethanol, the ear was allowed to air-dry, and placed against a disposable piece of filter paper for sampling. Sterile, disposable 2 mm diameter skin biopsy punches (Acuderm Inc., Ft. Lauderdale, Florida) were used to aseptically obtain ear biopsy samples. Biopsied ear tissue was removed from the biopsy instrument and placed in BSK media with forceps that were dipped in 95% ethanol and flamed between punches. Similarly, single peripheral and central ear punches were removed and separately placed in 100 μl of tissue extraction buffer (TEB) containing 50 mM Tris/HCl, 1 mM EDTA, 1% {w/v} Laureth 12 (PPG/Mazer Chemicals, Gurnee, Illinois, USA) for PCR. In all cases, there was at least 2 mm of unbiopsied tissue between ear biopsy locations. The mouse's ear was relocated on the filter paper between punches to avoid cross-contamination.

Ear-tissue samples were incubated in BSK media at 34 C for up to 6 wk and were examined by dark field microscopy twice weekly for the presence of spirochetes. Cultured spirochetes were identified as *B. burgdorferi* by immunofluorescence assay (IFA) using monoclonal antibody H5332 (provided by A. G. Barbour, University of Texas Medical School, San Antonio, Texas, USA), specific for outer surface protein A (OspA) (Barbour et al., 1983). Spot slides for IFA were prepared from spirochetes harvested from BSK media by centrifugation and washed twice in PBS. The slides were reacted with a 1:100 dilution of H5332 in PBS, at 37 C for 1 hr, washed, reacted with a 1:30 dilution of FITC-conjugated goat anti-mouse immunoglobulin G (IgG) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland), and observed for fluorescence at 400×. Additionally, detection of OspA coding sequences by PCR using primers BAE-1 and BAE-2 was used to confirm the identity of spirochetes as *B. burgdorferi* is described by Hofmeister et al. (1992). To detect OspA sequences in ear tissue, biopsy samples were incubated overnight in TEB at 37 C and placed in a boiling water bath for 10 min prior to amplification of a 10 μl sample. The TEB alone was used as a negative control following each animal's group of ear biopsy samples and was evaluated in the same manner as that used for tissue samples. Reaction mixtures were prepared and the samples were added with positive displacement pipettors in a laminar flow hood in which PCR product was not handled. Distilled water was used in place of template DNA after every tenth specimen to serve as a negative PCR control. The PCR products were detected by visualization of the expected amplification product on agarose gels and by Southern hybridization with a ^{32}P-labeled oligonucleotide, as described of Hofmeister et al. (1992).

To determine whether the agreement between culture and PCR depended upon obtaining samples from the same or from different ears, both ears were sampled (one ear for culture and the other ear for PCR) in 29 mice and a single ear was sampled for both assays in 21 animals. Culture of *B. burgdorferi* was considered to be the standard for infection with the organism in individual mice. For comparisons of culture and PCR in detecting *B. burgdorferi* by ear sampling location, the location was des-

ignated as being infected if either replicate ear biopsy was culture-positive. The frequency of detection of *B. burgdorferi* by peripheral versus central biopsy was compared by contingency table analysis using Fisher's exact test separately for both culture and PCR methods (Fleiss, 1981). The agreement between culture and PCR on detection of *B. burgdorferi* by tissue source was determined by calculating Kappa (range −1.0 to +1.0) and its standard error (Fleiss, 1981), and was characterized as "excellent" (Kappa > 0.75), "moderate" (Kappa ≥ 0.40 and ≤ 0.75), or "poor" (Kappa < 0.40).

RESULTS

Borrelia burgdorferi was isolated by culture from one or more organ or ear tissue samples from 33 (66%) of 50 captured *P. leucopus*. From the 66 pairs of ear biopsy samples, four cultures were discarded due to overgrowth of bacterial contaminants: two peripheral and two central samples removed from three mice. Overall, agreement between the paired ear biopsy samples on detection of *B. burgdorferi* by culture at a biopsy location was observed in 44 (71%) of the remaining 62 paired samples. All cultured spirochetes were positive for OspA by IFA with monoclonal antibody H5332; thus they were *B. burgdorferi*. In addition, OspA sequences were amplified in all isolates by PCR primers BAE-1 and BAE-2 and were specifically hybridized with {^{32}P}-labeled oligonucleotide BAE-3 (data not shown). In no case were negative PCR controls, included in PCR analysis of cultured spirochetes or analysis of ear biopsy samples, positive for *ospA*. In mice in which one or more organ sample was culture-positive, *B. burgdorferi* was detected by both culture and PCR of ear biopsy tissue in 30 (94%) of 32 mice and by PCR only in two (6%) of 32 mice. From the 32 mice with one or more organ culture-positive for *B. burgdorferi* the organism was cultured from 20 (63%) kidney, 31 (97%) spleen, and 26 (82%) bladder samples. Additionally, the organism was detected by both culture and PCR of ear biopsy tissue in one mouse in which spirochetes were not detected by organ culture.

Overall, *B. burgdorferi* was detected by culture in 29 (88%) peripheral ear biopsy samples and 28 (85%) central samples obtained from the 33 mice infected with *B. burgdorferi* (Table 1); this difference was not significant ($P = 0.71$). Similarly, the difference in the frequency of culture-positive peripheral and central ear samples was not significant in mice sampled from both ears or a single ear (Table 1). By PCR, *B. burgdorferi* was detected in 24 (73%) of 33 of peripheral and in all 33 central ear biopsy samples obtained from the infected mice (Table 1). This difference in the overall frequency of detection of *B. burgdorferi* by PCR in peripheral and central ear samples was significant by Fisher's exact two-tailed ($P = 0.002$). By sample group, the organism was detected by PCR in 11 of 17 peripheral and all 17 central ear samples obtained when both ears were sampled, and 13 of 16 peripheral and all 16 central ear samples when a single ear was tested (Table 1). This resulted in a significant difference in frequency of PCR positive samples between peripheral and central samples by Fisher's exact two-tailed ($P = 0.003$) when both ears were sampled, but not when a single ear was sampled ($P = 0.22$).

When the results of both assays were compared in mice sampled in both ears, a single ear, or overall; moderate agreement between assays (Kappa ≥ 0.40 and ≤ 75) was observed in peripheral ear samples (Table 2). In contrast, excellent agreement (Kappa > 0.75) was observed in central ear samples when results of culture and PCR were compared across both sampling groups and overall. Discordant results in peripheral samples included detection of *B. burgdorferi* in two samples by PCR only and in seven samples by culture only. In contrast, in central ear samples the organism was detected in five samples by PCR only and in no samples by culture only.

DISCUSSION

Based on our results, we propose that culture and PCR of ear biopsy samples are

TABLE 1. Detection of *Borrelia burgdorferi* by culture and the polymerase chain reaction (PCR) in ear punch biopsy samples removed from the peripheral and central part of the ears of naturally infected *Peromyscus leucopus*.

Group	Number of mice tested	Culture[a]		PCR	
		Ear biopsy location		Ear Biopsy location	
		Peripheral	Central	Peripheral	Central
Both ears	17	14 (82)[b]	14 (82)	11 (65)	17 (100)[c]
Single ear	16	15 (94)	14 (88)	13 (81)	16 (100)
Overall	33	29 (88)	28 (85)	24 (73)	33 (100)[c]

[a] Culture result of either replicate ear biopsy sample taken from both locations.
[b] Number positive (percent positive).
[c] $P < 0.05$ based on Fisher's exact probability test comparing the number of PCR positive central biopsy samples with the number of PCR positive peripheral samples.

highly sensitive methods for detection of *B. burgdorferi* in naturally infected mice. Further, while detection of *B. burgdorferi* by culture may be independent of the site from which the sample is removed, the organism may be detected by PCR with greater sensitivity in samples removed from the central portion of the ear. The overall sensitivity of culture of ear biopsy samples observed in both peripheral and central samples was similar to that estimate observed previously for culture of ear biopsy samples removed from naturally infected mice (85%) (Hofmeister et al., 1992). Our observations are in agreement with other reports in which culture of ear biopsy samples was reported to be a highly sensitive method of detection of the organism in experimentally infected laboratory mice (Barthold et al., 1992; Moody et al., 1994).

For this study we used culture results from internal organs for determination of the infection status of individual wild-caught mice. Detection of *B. burgdorferi* by culture of internal organs provided evidence both that an active infection with the organism was present at the time the mouse was sampled and that the infection was present for a period of time sufficient for the organism to disseminate from the site of tick feeding. Detection of *B. burgdorferi* by both culture and PCR in one mouse in which internal organs were culture-negative is further evidence of the sensitivity of the ear biopsy method. We hypothesize that this animal only recently

TABLE 2. Agreement between culture and polymerase chain reaction (PCR) for detection of *Borrelia burgdorferi* in ear biopsy samples removed from the peripheral and central part of the ears of wild caught *Peromyscus leucopus* by biopsy location.

Groups of mice	Number tested	Number positive			Number negative	
		Both methods	PCR only	Culture only	Both methods	Kappa (SE)
Peripheral biopsy location						
Both ears	29	10	1	4	14	0.66 (0.181)[a]
Single ear	21	12	1	3	5	0.57 (0.213)[a]
Overall	50	22	2	7	19	0.64 (0.139)[a]
Central biopsy location						
Both ears	29	14	3	0	12	0.79 (0.084)[a]
Single ears	21	14	2	0	5	0.78 (0.212)[a]
Overall	50	28	5	0	17	0.79 (0.138)[a]

[a] $P < 0.01$ for the test of Kappa = 0 versus Kappa > 0.

had been infected with *B. burgdorferi*, and the parasite had not fully disseminated from the site of tick feeding to establish systemic infection.

Other methods for detection of infection with *B. burgdorferi* which do not require killing the mouse include culture of blood, detection of the spirochete by tick xenodiagnosis (Levine et al., 1985), and culture of spirochetes obtained by needle aspiration (NieLin and Kocan, 1993). However, culture of blood for spirochetes has been reported to be much less sensitive than culture of other tissues in naturally (Anderson et al., 1985, 1987) and experimentally infected mice (Barthold et al, 1992; Moody et al., 1994). Detection of the organism by xenodiagnosis requires the maintenance of a tick colony and the removal of the captured rodent to a laboratory for several days, thus making it an impractical technique for field studies. The needle aspiration method has been reported to have a sensitivity of 100% for detection of *B. burgdorferi* at 40 days postinoculation in a small group of experimentally infected mice (NieLin and Kocan, 1993); this is equivalent to the sensitivity for culture or PCR in ear biopsy samples from experimentally infected mice (Hofmeister et al., 1992). However, the needle aspiration method may not be practical for mice sampled in the field. Thus, the ear biopsy method, in our opinion, is the most efficacious and practical method for detection of the organism in both naturally and experimentally infected mice. Additionally, in longitudinal studies, repeated ear biopsy of individual animals has few demonstrable deleterious effects on an animal. Experimentally infected hamsters have been repeatably biopsied for 20 wk (Sinsky and Piesman, 1989) and we have removed as many as 10 ear biopsy samples from wild-caught *P. leucopus* followed longitudinally for over 14 mo. The life expectancy of biopsied animals is similar to that expected for animals captured and released repeatedly (E. K. Hofmeister, unpubl.).

Detection of *B. burgdorferi* by culture in this group of naturally infected *P. leucopus* was independent of the location from which the sample was removed. This result is in contrast to the findings by Sinsky and Piesman (1989) that detection of *B. burgdorferi* in ear biopsy samples removed 30 days post-infection from two hamsters was less efficient in samples removed from the periphery of the ear compared to those obtained from the center. Sinksy and Piesman (1989) also observed that the density of spirochete growth in culture increased as ear biopsy tissue was removed closer to the base and the center of the ear. We did not observe a difference by ear biopsy location in either the density of spirochete growth nor in the number of days in culture prior to detection of the organism by dark-field microscopy. In contrast to our culture results, we found that the central part of the ear may be more sensitive for detection of *B. burgdorferi* by PCR as the organism was detected in significantly more central biopsy samples overall and in mice sampled from both ears (Table 1). While this relationship between tissue source and detection of *ospA* by PCR did not attain statistical significance in mice sampled from one ear only, a similar trend was observed in which *ospA* was detected by PCR in more samples removed from the central portion of the ear obtained from mice sampled in a single ear only.

We observed a higher level of agreement between culture and PCR assays in biopsy samples removed from the central location of the ear, as compared to the peripheral. While the 95% confidence intervals for the peripheral and central Kappa values overlap, this conclusion also was based on a comparison of the discordant results on detection of the organism by sampling location; in peripheral biopsy samples, *B. burgdorferi* was detected in the majority of discordant samples by culture. In contrast, among central samples, *B. burgdorferi* was detected in the majority of discordant samples by PCR (Table 2). We also observed the same level of

agreement between culture and PCR on detection of *B. burgdorferi* in central ear biopsy samples whether the samples were removed from the same or separate ears. This result is particularly useful in designing field sampling protocols in which the mouse might be sampled by ear biopsy for both detection methods and ear tagged at the same time.

Our results may be partially explained by the fact that culture detects only viable organisms whereas PCR is capable of detecting both viable organisms and isolated nucleic acid sequences. The central part of the ear may contain spirochetes which have migrated to that location from the site of tick feeding. Spirochetal cellular debris also may be channeled from the site of acute infection through the central part of the ear along the central vessels. Finally, spirochetes may have disseminated to the central part of the ear through the vascular system following systemic infection. *Borrelia burgdorferi* may have a specific trophism for the skin in chronically infected rodents; this, in turn, may depend on the distance from the centrally located major vessels in the ear. In chronically infected laboratory mice experimentally inoculated with *B. burgdorferi*, the culture of ear biopsy samples, as opposed to culture of internal organs, resulted in the most consistently sensitive detection of the organism (Barthold et al., 1993).

We recommend that samples be removed from the central part of the ear in studies in which PCR of ear biopsy samples is used to detect *B. burgdorferi* in wild-caught *P. leucopus*. This protocol will maximize the sensitivity of detecting infection and will achieve the highest concordance between assays in studies in which both culture and PCR are conducted in parallel.

ACKNOWLEDGMENTS

This work was supported by R55 A130042 from the National Institute of Allergy and Infectious Diseases and by a Faculty Development Award to James E. Childs. The authors are indebted to J. Piesman, Centers for Disease Control and Prevention, for his comments on an earlier draft of the manuscript.

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Received for publication 1 June 1994.