artesunate [6]. On the other hand, the potentially multigenic mechanism generally associated with quinoline antimalarial drugs offers hope for a slower development of resistance to lumefantrine.

We presently favor the hypothesis that the selection of the pfmdr1 86N mutation is just the first step in a multistep process, as opposed to being a genetic background allowing the establishment of 1 mutation that will lead to complete resistance. The exception to this view might be the selection of pfmdr1 duplications in the carriers of the 86N mutation, as has been observed, in Thailand, in studies of exposure to mefloquine [7]; however, preliminary data from pfmdr1 copy-number analysis currently being finalized in our laboratory do not support this hypothesis.

Another potentially favorable factor that might prevent the early selection of resistance to the artemether-lumefantrine combination is the demonstrated synergy between these 2 drugs [8], which acts most effectively against parasites carrying the pfmdr1 86N mutation [9], an observation that concords with the absence of any detectable selection of the pfmdr1 86N mutation in our study of recrudesences. In summary, we view the pfmdr1 86N mutation only as a tolerance-specific mutation, the parasite population’s first reaction to the new drug, a reaction that may lead to future resistance.

The previous comment leads us to our only clear disagreement with Hastings and Ward—the disagreement with their statement that the 86N mutations “were presumably already present at significant frequencies, probably because of local use of structurally related antimalarial therapies.” The pfmdr1 86N mutation is present in all malaria settings where it has been investigated, with the N86Y mutation being 1 of the 5 natural nonsynonymous single-nucleotide polymorphisms known to occur in this gene. If any selection occurred in the malaria setting studied, it would be expected to have favored the 86Y mutation, which is associated with resistance to chloroquine [10], a fact that would explain the higher (76%) pretreatment prevalence of this latter mutation. This view is indirectly supported by unpublished data from our laboratory that show a high prevalence of the 86N mutation among parasite isolates collected in central Liberia during the late 1970s, before the mass implementation of antimalarial drugs in that region [11] (J. Ursing, personal communication). It should also be emphasized that, in Zanzibar, Coartem is employed as a second-line treatment and therefore has been used on only a limited scale.

There is consensus that combination therapy in general—and ACT in particular—is the way forward in antimalarial chemotherapy. Maybe the optimal future combination will be a 3-drug ACT including 2 intersynergistic quinoline drugs with similar, relatively long half-lives, protecting each others’ efficacies after the fast elimination of the artesininine derivative. Additionally, the expected complex multigenic mechanism needed for quinolone-based antimalarial resistance would be expected to slow down the emergence of this resistance to these drugs. Finally, we agree that efficient monitoring of resistance is essential, a requirement that clearly suggests an urgent need for increased support of research leading to the definition of molecular markers of resistance to the partner drugs in combination therapy.

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References

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The Role of Chlamydia pneumoniae in Multiple Sclerosis: Real or Fictitious?

To the Editor—The possible association between Chlamydia pneumoniae infection and multiple sclerosis (MS) was first described in a case study by Sriram and colleagues at Vanderbilt University Medical Center (VUMC), which was then followed by a study of a series of patients from VUMC in whom the researchers reported that the organism was identified by culture and polymerase chain reaction (PCR) [1, 2]. The results of subsequent studies performed by a number of other groups have...
been conflicting, finding *C. pneumoniae* DNA in 0% to >80% of cerebrospinal fluid (CSF) samples from patients with MS and in 0%–20% of CSF samples from patients with other neurologic diseases [3]. This discrepancy in results is similar to inconsistencies in findings reported for the association between *C. pneumoniae* infection and atherosclerosis; some studies have identified the organism by PCR and/or immunohistochemical (IHC) staining in up to 100% of atheromatous tissues tested, but other studies have not confirmed these findings [4, 5]. These discrepancies may indicate methodologic differences, sampling error, or other unknown problems. Several multicenter studies have demonstrated major differences in methodologies and results, including significant inter- and intralaboratory variability in PCR testing when the same specimens were tested in different laboratories, even among those using the same assays [4–6].

The conflict between results reported in studies of the association between *C. pneumoniae* infection and MS may also be the result of methodologic problems. In 2002, Kaufman et al. [7], in an effort to deal with the issue of interlaboratory differences in methods used to detect *C. pneumoniae* in patients with MS, prospectively collected 30 CSF samples from patients with MS and 22 CSF samples from patients with other neurologic diseases; these samples were sent to laboratories at VUMC, Johns Hopkins University (JHU), and Umeå University (UU) in Sweden and, subsequently, to the Centers for Disease Control and Prevention (CDC). None of the CSF samples was found to be positive by PCR at JHU, UU, and the CDC, but 73% of the CSF samples from patients with MS and 23% of the control samples were found to be positive by PCR at VUMC. Possible reasons for these discrepant results were discussed in the published article and included (1) poor sensitivities of the 3 different and well-validated PCR assays used by JHU, UU, and the CDC and (2) specificity problems with the PCR assay used by VUMC. Since the results of only 1 of 4 different PCRs from 1 laboratory were discrepant, of interest was the specificity of the primer sequences used in the VUMC PCR—that is, were they specific for only *C. pneumoniae*? The primer sets used by VUMC in the multicenter study and the sets used in previous studies performed in that laboratory were analyzed and were found to have high sequence similarity to human DNA, as determined by BLAST search and amplification of human DNA [8]. These findings suggested that the primers used were not uniquely specific for *C. pneumoniae*.

The present article by Sriram et al. [9] should be read in this context. The authors report identification of *C. pneumoniae* in CSF and brain tissue from a high proportion of patients with MS, as detected by both nested PCR and IHC staining. Two different sets of PCR primers were used, directed at the *C. pneumoniae* major outer membrane protein (MOMP) and 16s RNA genes. However, the primer sets for MOMP used in the present study were the same as the ones VUMC used in the multicenter study and later analyzed [7, 8]. There are numerous issues regarding the specificity of the PCR used for the detection of *C. pneumoniae* in this study, as well as other PCR data generated by this method [8]. These issues include the specificity of the primer sequences, the low temperature used for the annealing reaction, and the questionable application of a nested touchdown assay format. Primers for both PCR assays used in the study target homologous human DNA sequences in addition to *C. pneumoniae*, according to BLAST as of January 2005, and, thus, may lead to nonspecific PCR product formation. Thus, it is likely that primers used in both PCRs generated nonspecific amplification products.

The format chosen for both PCR assays also poses a specificity problem. Both PCRs were based on the touchdown technique, which should use a high (e.g., 65°C) annealing temperature during the first cycles to increase specificity, followed by decreasing annealing temperatures for efficient amplicon amplification. However, the PCR used by Sriram et al. [9] began the touchdown protocol with annealing at 58°C, followed by decreases in temperature to 48°C. The above-described primer pairs in combination with this un-exacting touchdown protocol may also lead to a nonspecific PCR product [8].

Considerable difficulty has also been experienced with antigen detection, because of nonspecific background staining and the morphological heterogeneity of *C. pneumoniae* elementary bodies [10]. Human and chlamydial heat-shock proteins have highly conserved and homologous antigenic sites. Consequently, such antigens may interfere in tissue diagnostics, resulting in cross-reactions and false-positive results [11]. In the Sriram et al. study, the authors report positive results based on IHC detection methods for 7 of 20 samples of formalin-fixed brain tissue from patients with MS, by use of 3 *Chlamydia* genus-specific antibodies, but the positive results could not be confirmed by staining with 2 different *C. pneumoniae* species-specific antibodies. The authors used mouse lungs infected with *C. pneumoniae* as a “positive” control. Although such infected mouse lung cells can be used to establish the sensitivity of the IHC assay, they cannot be used for establishing the specificity of an assay intended for detection of *C. pneumoniae* in human tissue. The detection of putative *C. pneumoniae* DNA and antigens more frequently in the patients with MS than in control subjects is also probably due to nonspecific DNA sequences that may be present more frequently in MS, including lymphocytes and other cells in the CSF and increased synthesis of immunoglobulins. The rate of detection of *C. pneumoniae* DNA in the control CSF samples is consistent with the background rate of contamination seen with nested PCR assays [6].

When in-house–produced laboratory methods are developed and implemented, as in the study by Sriram et al., it is im-
References


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Reply to Hammerschlag et al.

To the Editor—In their letter, Hammerschlag et al. [1] raise 2 major issues to which we would like to respond. These issues are the specificity of the polymerase chain reaction (PCR) assays and of the immunohistochemical (IHC) staining we used in our study [2].

The comments pertaining to the lack of specificity of the primer set and PCR amplification procedure for amplification of the major outer membrane protein (MOMP) gene are identical to those the same authors have raised previously; we have addressed them in the past [3]. There is no single accepted method for detection of Chlamydia pneumoniae DNA and, therefore, in-house assays that are verified internally are being used. We recognize the problems inherent in using nested PCR assays. To increase the specificity of our nucleic acid–based amplification assays, we required amplification with primers for 2 different C. pneumoniae genes to designate a sample as positive [4].

We must also point out that the conditions of our touchdown technique for the amplification of the 16s RNA gene began with an annealing temperature of 60°C—and not 58°C, as the authors indicated—a temperature that was similar to the beginning temperature (62°C) in experiments published by the authors and not 65°C, as they now suggest [5].

Regarding the use of IHC staining, Hammerschlag et al. state that the initial positive control should be human tissue infected with C. pneumoniae. We have, however, followed the recommendation on IHC staining that 3 of the authors of the letter suggested in their review article and used infected tissue from experimental animals as our internal positive controls [6].

Hammerschlag et al. also comment that the anti-chlamydial hsp60 antibody that was used for IHC staining can cross-react with human hsp60 antigens, citing the work of Ochiai et al. to support their view [7]. The data in this article, however, do not support this assertion. The anti-hsp60 antibody that we used does not cross-react with human hsp60. The staining of chlamydial antigens obtained with the 3 different antibodies we used was similar in pattern and distribution and was not seen with the isotype-matched control antibodies. Thus, we believe our findings are unlikely to represent false-positive staining.

Hammerschlag and colleagues have, in prior studies, not detected C. pneumoniae in CNS tissue of patients with multiple sclerosis (MS), arguing for their absence in MS [8]. Their study did not anticipate the possibility that C. pneumoniae antigens are present predominantly in ependymal cells. Since their experimental design failed to address the possibility of distinct anatomic localization of Chlamydia antigens, their experiments proved unsuccessful. Furthermore, our findings showing an association between C. pneumoniae infection and MS are supported by the results of studies performed in laboratories in 4 different countries. At least 7 studies have shown C. pneumoniae DNA to be present in the CSF of patients with MS and absent in the CSF of control individuals [9]. The more recent finding of C. pneumoniae RNA in the CSF of patients with MS also

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