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Expert Review of Anti-infective Therapy

ISSN: 1478-7210 (Print) 1744-8336 (Online) Journal homepage: informahealthcare.com/journals/ierz20

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To cite this article: Sam R Telford III, Linden T Hu & Adriana Marques (2014) Is there a place for xenodiagnosis in the clinic?, Expert Review of Anti-infective Therapy, 12:11, 1307-1310, DOI: 10.1586/14787210.2014.966084

To link to this article: https://doi.org/10.1586/14787210.2014.966084

Is there a place for xenodiagnosis in the clinic?

Expert Rev. Anti Infect. Ther. 12(11), 1307–1310 (2014)

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Whether *Borrelia burgdorferi,* the causative agent of Lyme disease, can persist after antibiotic therapy is an area of ongoing controversy. In animal models, *B. burgdorferi* DNA can be detected in tissues after antibiotic therapy as well as by using the natural tick vector to acquire the organism through feeding (xenodiagnosis). Vector arthropods have been successfully used in xenodiagnosis to describe the etiology of infections such as malaria, typhus and Chagas disease. Our recent safety trial of xenodiagnosis demonstrates that ticks may be successfully fed on patients and may help determine the biological basis for post-treatment Lyme disease syndrome.

Our recent study [1] demonstrated the safety and proof of concept for using xenodiagnosis, the feeding of a natural arthropod vector on a patient to detect evidence of infection, as a tool for probing the causes of post-treatment Lyme disease syndrome (PTLDS). We review the history of xenodiagnosis, recapitulate some of the major features of the PTLDS study and discuss the possible utility of this classical biological assay in the clinical setting.

History of xenodiagnosis

Xenodiagnosis has provided definitive evidence for the etiology of diverse vector-borne infections, including malaria, yellow fever, epidemic typhus and Chagas disease. Ross [2] fed mosquitoes on malaria patients and detected early stages of plasmodial development, later fully elucidated by Grassi and coworkers, also using xenodiagnosis [3]. Uninfected body lice were used in human xenodiagnosis studies to definitively identify *Rickettsia prowazekii* as the etiologic agent of epidemic typhus [4], as well as for confirming Zinsser's conjecture that Brill Zinsser disease comprised recrudescent typhus [5]. As a by-product of the Typhus Commission work [4], trench fever was also determined to be due to a rickettsia-like bacterium (now known as Bartonella quintana) transmitted by body lice. Such studies were research efforts in the clinical context of determining the etiology or describing the life cycles of the agents of burdensome diseases.

Xenodiagnosis was first proposed as a means of confirming a clinical diagnosis by Emile Brumpt in 1914 [6], particularly for Chagas disease, and indeed became the gold standard in many Latin American countries, achieving sensitivities of one trypanosome in several hundred microliters of blood. The utility of xenodiagnosis for Chagas disease was due to the sparse peripheral blood parasitemias in patients, particularly those who were chronically infected. Currently, xenodiagnosis for Chagas disease is being widely supplanted due to the expanded availability of PCR or antigen detection methods, as well as the general difficulties of maintaining a laboratory colony of bugs and feeding them on patients. Xenodiagnosis has not otherwise been used for clinical diagnosis.

Xenodiagnosis has long been used by medical entomologists in Lyme disease research to provide definitive evidence of a wild host's infection status [7]. Because theoretically only one spirochete is sufficient to infect a tick, the feeding of a few dozen larvae, which would each imbibe 20 µl or so of tissue fluids, would provide remarkable sensitivity that is not matched by PCR. There is

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KEYWORDS: *Borrelia burgdorferi* • clinical trial • *Ixodes dammini* • Lyme disease • xenodiagnosis

 10.1586/14787210.2014.966084 ISSN 1478-7210

evidence that spirochetes migrate toward tick saliva, and thus would be attracted into the tick feeding sites [8]. In contrast, PCR would only sample those spirochetes that were present in the skin in an unenhanced density. Finally, transmissibility to new uninfected ticks provides evidence for perpetuation; active motility is needed for *Borrelia burgdorferi* to colonize ticks. Thus, theoretical considerations suggest that feeding ticks might be the most sensitive and biologically relevant assay for infection due to viable spirochetes; larval cohorts were more likely to ingest a full repertoire of spirochetal clones relative to that detected from dermal biopsies of the experimentally infected mouse hosts [9].

The problem of Lyme disease

Lyme disease is a bacterial zoonosis transmitted by ticks in the *Ixodes persulcatus* species complex across the Holarctic. The spirochetes that cause Lyme disease are part of the *B. burgdorferi* sensu lato (s.l.) group. Three genospecies are the most commonly associated with human infections: *B. burgdorferi* sensu stricto, which causes disease in North America and Europe (hereafter referred as *B. burgdorferi*), *Borrelia afzelii* and *Borrelia garinii*, which occur in Europe and Asia. *B. burgdorferi* is delivered to the dermis by the prolonged feeding of a tick. Relatively few spirochetes replicate within the skin and migrate outward from the site of inoculation, thereby producing the pathognomonic erythema migrans rash. The spirochetes disseminate via the draining lymphatics or hematogenously, but never attain large densities in the lymph or blood. Direct evidence of the infection by culture and PCR has low sensitivity in humans outside acute illness or synovial fluid in Lyme arthritis. The available antibody-based assays cannot be used to determine successful eradication of the organism, as the current serologic testing for antibodies to *B. burgdorferi* may remain positive for years after therapy and declines very slowly, making it essentially useless for confirmation of successful therapy [10].

In animal models, skin is usually culture or PCR positive for the duration of infection. Dermotropism is biologically consistent, given that for there to be spirochetal perpetuation (continued generations within natural populations), a tick must acquire infection and pass it on during a subsequent blood meal. Ticks do not cannulate vessels, but feed on pools of lymph, blood and extravasated fluids from the ground substance of the dermis; this interface is modulated by an impressive tick salivary pharmacopeia, including anti-inflammatory, antihemostatic and immunosuppressive molecules [11]. Hence, Lyme disease spirochetes have specifically adapted to the dermal interface between ticks and mammalian hosts.

Many studies have shown that Lyme disease is treated successfully with antibiotics in the majority of cases and that patients with objective evidence of treatment failure are rare with the currently recommended regimens [12,13]. Patients with late manifestations can have a slower response to therapy, sometimes taking weeks or months to recover [14,15]. A minority of patients treated for Lyme disease will have persistent or relapsing non-specific symptoms (such as fatigue, musculoskeletal pain and cognitive complaints) after receiving an adequate course of antibiotic therapy, a condition that is called PTLDS [10]. The biological basis for the continued illness in PTLDS patients remains to be explained. One hypothesis is that viable spirochetes or their remnants may persist in these patients.

Evidence for persistence of *B. burgdorferi* s.l.

In the absence of treatment, the agent of Lyme disease can establish long-term, persistent infections in multiple animal species, including humans. *B. burgdorferi* s.l. has been recovered from the skin lesions of acrodermatitis chronicum atrophicans (a late manifestation of Lyme disease) patients as long as 10 years after the original infection [16]. The first evidence that *B. burgdorferi* or its remnants may persist following administration of antibiotics was described for dogs [17,18], although it is possible that their treatment regimen was not sufficient [19]. Xenodiagnosis using subadult deer ticks (*Ixodes dammini* or *Ixodes scapularis*) demonstrated the persistence of *B. burgdorferi* or its remnants after antibiotic treatment in mice [20–23] and non-human primates [24,25]. However, *B. burgdorferi* could not be reliably cultured from antibiotic-treated animals.

Safety of human xenodiagnosis for analyzing PTLDS

We developed procedures for human xenodiagnosis using uninfected larval deer ticks and determined the safety of the protocol [1]. Xenodiagnosis was performed in 36 participants, and seven participants underwent more than one procedure. Participants who underwent xenodiagnosis included 10 patients with high C6 antibody levels, 10 patients with PTLDS, five patients with acute erythema migrans after completion of antibiotic therapy, 1 patient with erythema migrans on therapy (who also underwent xenodiagnosis after antibiotic therapy) and 10 healthy volunteers.

A specific pathogen-free (SPF) colony of *I. dammini* was used for the xenodiagnostic studies. These ticks comprise a closed colony and are maintained on SPF rodent hosts. All larval batches were screened by PCR for the presence of all zoonotic agents known or alleged to be transmitted by *I. dammini* (*B. burgdorferi*, *Babesia* spp., *Anaplasma phagocytophilum, Borrelia miyamotoi*, *Bartonella* spp., *Rickettsia* spp., deer tick virus, orbiviruses). As an additional measure of safety, aliquants of larvae from each batch intended for use in xenodiagnosis were allowed to feed on severe combined immunodeficiency mice, which were monitored for signs of disease. Even with this extensive testing, it is possible that the ticks could transmit a new, unknown infectious agent. Thus, this possibility was explained during informed consent, and participants were asked to report any unexplained signs and symptoms for 30 days after removal of the ticks. We found no evidence for an unrecognized pathogen in our Phase I study.

From 25 to 30 SPF tick larvae were applied to each patient, and eventually we developed a protocol that allowed us to recover 30–50% of these as engorged ticks from each. Xenodiagnosis was well tolerated. All subjects successfully completed the tick placement (which took 4–5 days for completion) and

there were no withdrawals during the study. The most common adverse event was mild itching at the site, which was seen in 51% of subjects, with a median duration of 3 days. No serious adverse events were associated with the procedure. Although it was not a main endpoint of this study, we analyzed most of the ticks that we collected by multiple methods, and found two patients whom we considered positive by xenodiagnosis (DNA detection), a patient with erythema migrans who initiated antibiotic therapy at the same time that the ticks were placed and a PTLDS patient.

Future applications

Now that xenodiagnosis using SPF larval *I. dammini* ticks has been demonstrated to be safe and well tolerated, a larger trial is needed to determine whether a positive xenodiagnosis correlates with persistence of symptoms. The benefit of our xenodiagnostic study is that it offers a new tool to detect the continued presence of *B. burgdorferi* or its DNA in humans that has not been possible with other technologies. One caveat of human xenodiagnosis is that because we cannot ethically withhold treatment from a patient with early Lyme disease, we do not know the sensitivity of xenodiagnosis during times when spirochetes may be routinely found by skin biopsy culture or PCR of blood. This cannot be extrapolated from animal studies because the host–pathogen relationship may greatly differ; for example, mice are well known to sustain large spirochete burdens in many of their tissues. However, the sensitivity of xenodiagnosis in untreated early Lyme disease patients would be expected to be much different than in treated patients or patients with late disease where the bacterial load is much lower. As such, more important information is obtained by determining the association of positive xenodiagnostic testing with PTLDS and not sensitivity of the xenodiagnosis *per se*. In addition, the assays used in our safety trial relied on sensitive detection methods for *B. burgdorferi* DNA [26], thereby raising issues of interpreting the results of tests relative to spirochetal viability [27]. It is possible that our xenodiagnosis testing detects minute amounts of DNA/bacteria that have no clinical relevance. However, the association (or lack thereof) can now be determined by expanded clinical trials.

The effort needed to maintain an SPF tick colony, the mode of feeding ticks on patients, the need for multiple patient visits and the variability of the yield of engorging ticks because individuals vary in their attractiveness for feeding ticks, all make xenodiagnosis a challenging procedure. Given the logistical issues, xenodiagnosis for Lyme disease is unlikely to be used in routine practice, but it can offer researchers a tool to develop new tools or biomarkers for the disease. Perhaps, nanobots injecting recombinant versions of tick salivary proteins and sampling pools of blood and extravasated lymph in the dermal bite sites may be developed and may be more easily deployed in a clinical setting. We hope xenodiagnosis will also stimulate a closer look at the dermal vector–pathogen interface.

As with Lyme disease, xenodiagnosis is unlikely to be routinely used for virtually all other vector-borne infections, although it should be noted that there are specific and useful clinical indications for similarly burdensome procedures such as debridement of chronic wounds by maggots or treatment of venous congestion by application of medicinal leeches. The obvious infections for which xenodiagnosis may be useful would be those that are known to be vector-borne, maintain a chronic state and are characterized by sparse organisms that are very difficult to detect. In most such instances, the question would be one of basic biology, and, as with our study, xenodiagnosis can be a valuable tool for research. Xenodiagnosis might also be useful, as it has historically been, for identifying the etiology of illnesses that remain enigmatic but are associated with medically important arthropods, such as Masters' Disease/Southern Tick-Associated Rash Illness [28].

Financial & competing interests disclosure

SR Telford is a senior consulting scientist with Imugen, Inc., and consultant to Immunetics Inc. and Fuller Laboratories. The authors' research is funded by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases, the NIH Bench to Bedside program, and R21AI082436. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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