MOLECULAR DIAGNOSIS OF INFECTIOUS DISEASES:  
THE GOOD, THE BAD AND THE UGLY

Edward B. Breitschwerdt, DVM, DACVIM  
Chief Scientific Officer, Galaxy Diagnostics, Inc.  
Professor, Internal Medicine, NCSU, Raleigh, NC  
Adjunct Professor of Medicine, Duke University Medical Center

Based on research conducted at the Intracellular Pathogens Research Laboratory Center for Comparative Medicine and Translational Research College of Veterinary Medicine North Carolina State University Raleigh NC 27606

INTRODUCTION

One advantage of experience (in this case, a 30 year career as an academic internist) is the eventual realization that our collective efforts to manage illness in our patients have numerous limitations. There are obvious practical limitations, such as time, money, equipment and expertise (or the lack thereof), that confront clinicians and pathologists on a daily basis. However, there are somewhat less obvious limitations that relate to the sensitivity and specificity of diagnostic testing and the failure or inability to pursue unusual or unknown pathogens in our patients. Molecular diagnostic approaches have begun to facilitate a “modern day” revolution in our understanding of the interactions of multiple infectious agents, the complexity of disease expression induced by acute or chronic infection, and has resulted in the redefinition of “previously understood” diseases such as babesiosis and leishmaniasis. Although there is an increasing appreciation for the importance of co-infection or polymicrobial infection in animal and human diseases, there are considerable gaps in our current understanding of the interactions between viruses, bacteria, protozoa and fungi as interactive contributors to complex disease expression.

MOLECULAR DIAGNOSIS THE GOOD

Without question various molecular based tools have facilitated tremendous advances in diagnosis of infectious diseases. PCR amplification of organism-specific DNA sequences can be accomplished in a matter of hours and in most instances the detection of a PCR amplicon confirms active infection with a specific organism. This approach has distinct advantages over culture, which can require incubation times ranging from days, to weeks, to months depending on the organism. In most instances, approaches that provide rapid culture results select for only a few more easily grown organisms. Although serology or examination of the cell mediated immune response will remain an important component of infectious disease diagnosis, these tests identify evidence of immune recognition of a pathogen. Failure to confirm active infection, cross reactivity among various infectious agents and for an increasing number of infections (babesiosis, bartonellosis, leishmaniasis and others) the serological response to the organism may be minimal or non-existent, despite chronic, active infection are limitations to the interpretation of serological test results in a given patient.

In some instances, the advent of PCR and DNA sequencing has allowed for the detection, characterization and at times reclassification of previously unknown or uncultured organisms. A few examples include:
1. The contemporary discovery, rediscovery and reclassification of *Bartonella* species.

Historically, two members of this genus were classified as *Rochilamea* (*Bartonella bacilliformis*) and *Rickettsia* (*Rickettsia quintana* and subsequently *Rochilamea quintana*) species respectively. Although the genus *Rochilamea* is extinct, a recently discovered *Bartonella* species has been named *Bartonella rochilamea* in honor of the historical contributions of Henrique da Rocha-Lima. In 1992, only two *Bartonella* species (*B. bacilliformis, B. quintana*) were known to exist, whereas now there are greater than 22 *Bartonella* species or subspecies.

2. The reclassification of the *Hemobartonella* and *Eperythrozoon* in the genera *Mycoplasma*. As with *Rochilamea*, the *Hemobartonella* and *Eperythrozoon* genera no longer exist, as these organisms were reclassified as *Mycoplasma* species on the basis of 16S rDNA sequence similarity. As hemotropic *Mycoplasma* species have not yet been cultured, epidemiological and diagnostic studies were limited to visualization of the organism. PCR amplification and sequencing have facilitated a molecular-based reclassification of these organisms. In addition, PCR targeting specific *Mycoplasma* gene sequences has allowed for the more sensitive detection of these organisms in the blood of healthy and sick cats and dogs. As a result, depending on the population studied, a large percentage of healthy or sick cats can harbor *M. hemofelis, Candidatus Mycoplasma haemominutum* or *Candidatus Mycoplasma turicensis*.

3. The reclassification of *Ehrlichia equi* as *Anaplasma phagocytophilum*. In the United States, *E. equi* was discovered and initially defined by clinicians and researchers at the University of California at Davis as an acute febrile illness of horses that is accompanied by edema and a mild degree of thrombocytopenia. Progressively over the next 20 years, *E. equi* was found to induce an acute febrile illness, accompanied by thrombocytopenia in cats, dogs and human beings. Based upon 16S rRNA gene sequences derived from *Anaplasma, Ehrlichia* and *Cowdria* species *E. equi* was reclassified as an *Anaplasma* species. Subsequently, it has been recognized that various *A. phagocytophilum* strains induce disease in cats, dogs, horses and people throughout the northern hemisphere.

4. The reclassification of *Cowdria ruminantium* as *Ehrlichia ruminantium*. Historically, *C. ruminantium*, the causative agent of heartwater disease in cattle, has been an economically devastating tickborne infectious disease throughout much of Africa and some Caribbean Islands where *Amblyomma variegatium* ticks were imported on cattle shipped from Africa. Based upon the 16 rDNA sequence, *C. ruminantium* was reclassified as an *Ehrlichia* species. With the advent of PCR and DNA sequencing, evidence of *E. ruminantium* was reported in dogs and HIV infected people from South Africa. Although somewhat scientifically astounding, this observation may make phylogenetic and biological sense as it is now known that several members of the genus *Ehrlichia*, including *E. canis, E. ewingii* and *E. chaffeensis* can infect both dogs and people following transmission by a vector tick. Therefore, the use of more highly sensitive molecular-based detection assays has resulted in a breakdown of the perceived “species barrier” for several *Ehrlichia* species.

In some instances, the advent of PCR and DNA sequencing has allowed for the initial discovery (or rediscovery) of an entire genus of intravascular bacteria. A specific example includes the genus *Bartonella*:
1. In the United States Bartonella species were discovered in the early 1990s in association with efforts to characterize “unculturable,” silver-staining bacteria observed in HIV-infected individuals with bacillary angiomatosis and peliosis hepatis. Using 16S rRNA eubacterial primers and DNA sequencing, Dr. David Relman at Stanford University amplified sequences from BA tissues that were most similar to R. quintana and a previously unreported DNA sequence, which proved to be Bartonella henselae. Subsequent research confirmed that all cases of vasoproliferative peliosis hepatitis are associated with B. henselae infection, whereas bacillary angiomatosis has been associated with the isolation or molecular detection of B. quintana, B. henselae and most recently B. bovis.

2. After B. henselae was successful isolated from the blood of an HIV infected individual (B. henselae Houston I, ATCC type strain) it was determined that cats were the primary reservoir hosts from which this organism was transmitted to humans. Bartonella henselae, as opposed to Afipia felis, is now considered the primary if not the sole cause of human cat scratch disease. Infection in an immunosuppressed human population facilitated the initial recognition and molecular characterization of Bartonella species infection in the United States. Although the subsequent discoveries in the area of Bartonella research are too numerous to mention, B. quintana, historically associated with wars, famines and deprivation was found to be present in the U.S. for the first time in history. Our research group has subsequently described B. quintana infection in a cynomolgus monkey (Macaca fascicularis), in dogs with B. quintana endocarditis and in cats that putatively transmitted B. quintana to a woman by a bite. Detection of Bartonella species infections in human beings and cats also lead to the isolation of a novel Bartonella subspecies (B. vinsonii subsp. berkhoffii) from a dog with endocarditis.

3. During the past decade, an expanding number of Bartonella species (at least 22 species and subspecies) have been discovered in domestic and wild animals, which serve as the primary bacterial reservoirs from which Bartonella spp. are transmitted to human beings or to other non-host adapted animals via bites, scratches or following inoculation by a spectrum of arthropods (lice, fleas, sandflies, biting flies and potentially ticks). Cats, cows, dogs, mice, rabbits, rats and squirrels can experience chronic (months to years) blood-borne infections with host-adapted Bartonella species, with the potential for transmission by bites or scratches to people.

4. Our research group has emphasized studies that have resulted in the enhanced detection of Bartonella species in the blood of animals and humans using an optimized combined pre-enrichment culture medium and a highly sensitive PCR. Pre-enrichment culture followed by PCR amplification of bacterial specific genes has resulted in substantial improvement in the ability to detect Bartonella spp. in the blood of people or animals that have substantial arthropod exposure or occupational animal contact. By targeting multiple genes, we have also documented that dogs and people can be infected with more than one Bartonella species.

5. Bartonella species are now recognized as a cause of serious disease manifestations including arthritis, endocarditis, encephalitis, meningitis, hepatitis and lymphadenitis in dogs and people. It is very possible that these organisms represent an occupational risk for those individuals with extensive animal and arthropod exposure.

6. As most Bartonella species have been discovered in the past decade, there is still much to learn regarding routes of transmission and disease causing potential. Recently, we have
documented a high prevalence of *Bartonella* infection in the lymph nodes of healthy Golden retrievers and Golden retrievers with lymphoma. In this same study, we were unable to detect *Anaplasma* or *Ehrlichia* DNA in the blood or lymph nodes of the study population.

**MOLECULAR DIAGNOSIS THE BAD**

The basis of all molecular diagnostic testing is a cumulative genomic data base (Gen Bank), which stores and provides DNA sequences for the public good. The source of the sequence, pending publications and other information relative to the deposited sequence is cited in the data base. Unfortunately, there is substantial variation in the length and quality of the deposited sequence and the data that is provided relative to the source of the sequence. Although by definition a molecular based diagnostic test should be 100% specific, based upon the premise that a unique gene sequence is being targeted, this is not always true or feasible for technical reasons. A few specific examples:

1. The 16S-23S intergenic spacer region has proven to be a valuable target for the molecular-based diagnosis of *Bartonella* species infection. For the genus *Bartonella*, there is in most instances substantial variation among species within the ITS region. Therefore, using the ITS region as a molecular target would allow for both detection and speciation of the infecting *Bartonella* species in a single PCR reaction. When PCR primers were selected for this purpose by our laboratory and others, there *Mesorhizobium* (a plant adapted bacteria that is phylogenetically related to the genus *Bartonella*), ITS sequences had not been deposited in Gen Bank. As would be expected, this plant bacteria is frequently found in water and unfortunately can contaminate molecular grade water resulting in a false positive diagnostic test result (amplification of *Mesorhizobium* DNA that is of the same amplicon size as a *Bartonella* species). In both our diagnostic (Vector Borne Diseases Diagnostic Laboratory) and research laboratories (Intracellular Pathogens Research Laboratory) we have a saying: A band on a gel is only a band on a gel until a DNA sequence from that specific band is available for genus and species confirmation. The advent of real-time (or quantitative PCR) with the use of melt curves has improved, but has not eliminated the potential for cross priming and misinterpretation of a PCR result.

2. Due to the available sequences and the relative high degree of genomic conservation among these species, most diagnostic laboratories target the 16S rRNA gene by PCR for the diagnosis of *Anaplasma* or *Ehrlichia* infection. In our laboratory a single PCR reaction is used to document infection with either species. If a positive genus PCR result is obtained, then 5 independent PCR reactions are used to determine if the patient is infected with *E. canis, E. chaffeensis, E. ewingii, A. phagocytophilum* or *A. platys*. This allows us to determine the infecting species, which has clinical, epidemiological and zoonotic implications and allows for the detection of co-infection with more than one *Anaplasma* and *Ehrlichia* species. Unfortunately, all bacteria are believed to have one or more 16S rRNA genes, most of which have been conserved in bacteria for millions of years. When a diagnostic test targets a gene that is highly conserved among numerous bacterial species, cross priming can result in multiple bands or bands of an appropriate size for the targeted organism because of contamination of molecular grade water, the
Taq polymerase or the primers with bacterial DNA. Although a potentially valuable diagnostic tool, assays that non-specifically target the 16S rRNA gene (i.e. eubacterial primers) can be technically problematic for a number of reasons.

**MOLECULAR DIAGNOSIS THE UGLY**

The international genome data base (Gen Bank) is available to anyone with computer access. Therefore the sequences required for the design of a molecular diagnostic test are also available to anyone. Kits can be purchased for DNA extraction and thermocyclers used for PCR amplification are no longer cost prohibitive for many commercial laboratories. Unfortunately, technical expertise and rigid quality control is absolutely critical for the accurate performance of molecular diagnostic testing. There is no standardization or quality control testing among laboratories providing molecular diagnostic test results to the public. As is stated for other consumables, “Let the buyer beware!” In addition, many practicing veterinarians are not familiar with the strengths and limitations of this diagnostic approach, therefore assistance in the interpretation of both positive and negative test results is frequently required.

There are numerous other issues that complicate and challenge the current state of the art in molecular diagnostic testing. A few of these include:

1. An exceptionally well designed PCR assay that will reproducibly detect a low genome copy number (1-2 copies of an organism-specific gene target in a 50ul PCR reaction) may not work as efficiently or may not work at all if transferred to a new (same manufacturer) or different thermocycler (different laboratory or different thermocycler manufacturer). Most importantly, different reagent quality, for example the source of Taq polymerase, can influence PCR sensitivity by 100-1000 fold.

2. PCR contamination is a constant fear for the Director of a molecular diagnostic laboratory. Unfortunately, despite appropriate controls, it is impossible to prove that a given PCR amplicon obtained from a patient sample is not a function of PCR contamination in the laboratory. Although a serious concern, the use of negative controls (extraction control, PCR control) in conjunction with laboratory surveillance greatly minimizes this concern. For technical reasons, the advent of real-time PCR has also minimized this concern.

3. With the use of samples such as paraffin blocks, specific protocols must be followed to avoid DNA carry over during the collection and extraction process, which has resulted in the reporting of false positive PCR results and data misinterpretation in the literature.

It is increasingly clear that the use of genomic approaches will continue to enhance our ability to diagnose and manage infectious diseases. The use of microarrays has already demonstrated utility for some diagnostic applications and testing on an array can facilitate the simultaneous detection of multiple RNA or DNA sequences (i.e. multiple organisms) in a sample at the same time point. However, the sensitivity of microarrays may be less than optimal for detection of some infectious agents. Therefore, the validation process should incorporate the use of clinical samples to determine if detection by array is as sensitive as PCR amplification techniques. Efforts to detect bioterrorism agents will propel developmental efforts in molecular based
diagnostic testing and will enhance the availability and utility of approaches that can be used for the evaluation of patient samples.

REFERENCES


