REVIEW ARTICLE

Laboratory diagnosis of rickettsial infection

Stephen Graves[1], John Stenos[2], Nathan Unsworth[3] and Chelsea Nguyen[4]

Director[1], Senior Scientist[2], PhD Student[3], Scientist[4],
Australian Rickettsial Reference Laboratory,
Geelong Hospital, Geelong, Victoria, 3220, Australia

Abstract

Rickettsial diseases (including Q-fever) are difficult to diagnose both clinically and in the laboratory.

Serology is the main diagnostic modality for rickettsial diseases and micro immunofluorescence is the recommended serological method. However serology can be unreliable (negative) early in the illness when the patient presents for medical attention. This is when the first serum is usually taken. Serology on later sera (which may by now have become positive) is often not performed and thus the diagnosis is not confirmed by the laboratory.

Recently nucleic acid amplification (NAA) has been used in some (reference) laboratories and provides a diagnostic result during the patient's acute illness, before antibodies have developed. DNA detection by real time polymerase chain reaction (PCR) is extremely sensitive and specific. However, such tests are not widely available.

Tissue culture of rickettsiae is not a useful method of diagnosis because it takes too long, and is too risky for routine diagnostic laboratories. Direct staining for rickettsia is rarely used.

Keywords: Rickettsia, diagnosis, Q-fever

Introduction

Rickettsial diseases (including Q-fever) are worldwide in their distribution. Australia has all rickettsial groups (Graves 2002, 2004). Even New Zealand, which does not have endemic Q-fever, has endemic murine typhus *(Rickettsia typhi)* (Roberts 2001) and cat flea typhus *(R. felis)* (Kelly 2004). Thus doctors throughout the world are called upon to diagnose rickettsial infection. Sometimes they send patient specimens (usually blood) to a diagnostic pathology laboratory to assist them in arriving at a diagnosis. The laboratory has the task of confirming or rejecting the diagnosis of rickettsial infection. Often this is not easy and requires skilled laboratory scientists in an adequately resourced laboratory (Walker and Dumler 1995).

Correspondence to: S Graves
Australian Rickettsial Reference Laboratory
Geelong Hospital
Geelong Victoria Australia

The most difficult component of diagnosing a rickettsial disease occurs in the doctor's consulting room, or at the hospital bedside, where the doctor must first think of rickettsial disease and include it in the list of differential diagnoses being considered. The symptoms of rickettsial disease are not specific to rickettsial disease and may well be found in a number of other infectious diseases e.g. malaria, measles, meningococcaemia, typhoid fever, influenza, leptospirosis. If the doctor does not think of rickettsial disease as a diagnostic possibility, then diagnostic tests for rickettsial disease will not be requested. Consequently, the test will not be done in the laboratory and the diagnosis will be missed. When the microbiology or serology laboratory receives a serum with a request for "? rickettsial infection", the doctor has already thought of the diagnosis and this first hurdle has been crossed.

A. The use of serology for diagnosing rickettsial/Q-fever infection

A useful manual of serological methods is Heckemy and Rikihisa (2005). Although serology is the main diagnostic method used, it has pitfalls:

- Serum taken too early in the illness, before the patient's immune system has produced antibodies, will be negative. A second serum, to show possible seroconversion, is considered essential. Even if the first serum has a low positive titre, a second serum showing a rise in the titre is crucial as the positive first serum result may have been simply due to past rickettsial infection or even a serological cross-reaction.
- The patient may be infected with a rickettsia but not produce antibodies, owing to either early antibiotic treatment, which has abrogated the humoral response, or a genetically-based inability to mount an antibody response to that microorganism.
- The test being used in the laboratory may not be 100% sensitive (few tests are) and so it will miss some patients with genuine rickettsial disease. Different testing modalities have different sensitivities and specificities. Generally an assay with a high sensitivity has a low specificity i.e. it detects every genuine case plus a few non-cases as well. Tests with a high specificity often have a low sensitivity i.e. if the assay records the patient as positive, can be reasonably certain that they do have rickettsial antibodies, but not all genuine cases are detected.

Thus some enzyme immuno-assays (EIA) have high specificity but lower sensitivity, while some micro-immunofluorescence (IF) assays have high sensitivity but lower specificity. In the latter case the problem is by-passed by using a higher serum dilution for screening.

Sensitivity of an assay varies according to the duration of the infection in the patient and usually increases with later sera.

Weil-Felix (Proteus) agglutination test

The earliest serological method for detecting antibodies to rickettsiae (Weil and Felix 1916) was based on a cross-reaction between antibodies to rickettsia and bacteria of the *Proteus* genus (strains OX2, OX19 and OXK). It was observed that the serum of patients who had recovered from typhus (either epidemic typhus in Europe or scrub typhus in the tropical Orient) would agglutinate some strains of *Proteus*.

This is a classic serological cross-reaction, as *Proteus* and *Rickettsia* are not closely related phylogenetically but, by chance, have antigens in common, probably located in the lipopolysaccharide of their cell walls (Amano 1998).

While this test was historically very important, e.g. it helped distinguish typhoid fever from epidemic (louse-borne) typhus and it helped distinguish scrub typhus *(Orientia tsutsugamushi)* from epidemic typhus *(Rickettsia prowazekii)*, it lacks both sensitivity (it misses true cases of rickettsial disease), and specificity (it records as 'rickettsial' cases due to other causes e.g. *Proteus* spp pyelonephritis). It should no longer be used as better tests are now available.

Enzyme immuno assays (EIA)

This serological modality is very widely used in routine diagnostic laboratories. It has the advantage of being amenable to automation, which is important in most modern laboratories. Commercial kits are available, often from more than one manufacturer, giving the laboratory a choice of assays to use.

In Australia, PanBio produces a number of kits for determining antibodies to rickettsiae (including Q-fever).

EIA is based on detecting a coloured product of an enzymatic reaction, which is quantitatively related to the antigen-antibody reaction, which is itself related to the concentration of antibody in the patient's serum. The intensity of the coloured end product (measured as optical density units at a particular wavelength of light) is compared (as a ratio) with known negative sera.

Unfortunately , while a positive EIA result gives an indication of the level of antibody (i.e. "2.6" is more than "1.7"), it is not a titre. EIA results from different laboratory runs cannot be validly compared. Also, a patient with "2.6" units of colour does not have twice the antibody concentration of a "1.3" unit serum.

EIAs vary with respect to sensitivity and specificity, depending to a large extent on the antigen(s) used in the assay. Antigen may be native molecules (Field 2002) or recombinant molecules (Jiang 2003; Land 2002). They may be lipopolysac-

charides (Keysary and Strenger 1997). If the test is used as a screening assay, it must have good sensitivity. How good is good enough is a matter of opinion, but the authors think a screening assay should have a sensitivity of ≥ 95% i.e. it will miss only ≤5% of genuine cases. It should certainly not be below 90% i.e. in which case it will not miss >10% of all genuine cases. To use a screening test with a sensitivity <90% is to mislead your referring medical practitioners into thinking your assay will tell them if their patient has rickettsial disease while your laboratory is missing more than one in 10 genuine cases.

In a screening EIA one can afford to have a lower specificity, as any positives (genuine positives and false-positives) can be confirmed later with a second round, more specific assay such as micro-immunofluorescence. True positive are not missed but genuine cases are then confirmed by the second assay. False positives are screened out as negative by the second assay.

Some EIAs have been modified into a solid-phase assay (strip type 'bedside' test) involving antigen fixed onto a solid support (Broadhurst 1998; Ching 2001). Patient serum is run into it, by capillary action, producing a coloured band or spot. Such assays for rickettsial diseases may not be currently satisfactory but are likely to be more widely used in the future.

Micro-immunofluorescence (MIF)

This type of serological assay is currently considered to be the 'gold standard' for rickettsial serology (Philip 1976; Bozeman and Elisberg 1963; Peacock 1983). Other assays are often compared to it (Silpapojakul 1995; Coleman 2002, Field 2000). The antigen is the whole rickettsial cell, fixed onto a glass slide. In the case of Q-fever, the *Coxiella burnetii* cells are one of two types; phase 1 (virulent) – grown in animals; and phase 2 (avirulent) – grown in embryonated chicken eggs or tissue culture, so as to detect both the early antibody response (antibodies to phase 2 – protein antigens) and the later antibody response (antibodies to phase 1 – lipopolysaccharide antigens), which is associated clinically with chronic Q-fever.

As the patient's antibodies react with the rickettsial cell on the slide, the positive reaction is visualised as fluorescence using the UV microscope. This assay can distinguish different immunoglobulin classes (IgM, IgG, IgA) reacting with rickettsiae, by using specific – anti-human immunoglobulins (anti-IgM, anti-IgG and anti-IgA), produced in another animal and conjugated to fluorescein isothiocyanate (FITC).

Immunoperoxidase can be used to detect antigen-antibody reactions, without the need for a UV microscope (Bassett 1992).

A titre for a patient serum can be obtained by simply repeating the reaction at a series of doubling dilutions ($^1/_2$, $^1/_4$, $^1/_8$, etc) where the highest positive dilution is defined as the "titre" or end-point of the titration.

Such a titration should be repeatable, with an end-point within one doubling dilution in the same laboratory and within two doubling dilutions in a different laboratory.

Although very sensitive, MIF loses specificity at high serum concentrations (low serum dilutions) due to the large number of antibodies, of different specifities, in the patient's serum, that can cross-react with rickettsiae. This problem can usually be overcome by starting the titration at a serum dilution that excludes (through a diluting-out effect) those cross-reacting antibodies. In rickettsial MIF this has traditionally been a 1 in 64 serum dilution, although in our laboratory we currently use a 1 in 128 dilution.

Thus, all sera are screened at a $^1/_{128}$ dilution. Those that are negative are reported as 'negative'. Those that are positive are further titrated (to $^1/_{1024}$) and a end point determined. (If the last serum dilution, at $^1/_{1024}$, is still positive, the titre is reported at $\geq ^1/_{1024}$.) Serum that are positive at $^1/_{128}$, but no higher, are often difficult to interpret without a second (convalescent) sera. They could be:

- A genuine rickettsial infection, where the titre is low (but rising) as the infection is still in its early stages. In this case, the second serum will be a strong positive.
- A past-infection with rickettsia and the serum antibody levels have fallen to a low (titre $^1/_{128}$) level. In this case the second serum will have the same titre as the first serum. Titres tend to rise quickly, but fall slowly, as plasma cells (the antibody-synthesising cells) gradually die off.
- Cross-reacting antibody, where the patient has never been exposed to rickettsiae, but to some other antigenic stimulus (probably a microbe), that has stimulated the synthesis of antibodies that will react invitro with rickettsiae (a heterologous antigen).

It is not possible to distinguish between 2 and 3 without proceeding to another assay, such as a Western Blot (not commercially available for rickettsiae) or doing cross-absorptions on the sera with rickettsiae. Cross-reacting (non-specific) antibodies may remain whereas specific antibodies are absorbed out of the serum.

Micro-immunofluorescence assays available in Australia include those from PanBio, bio-Merieux and the Australian Rickettsial Reference Laboratory (ARRL). There has been no formal comparisons of these different assays.

Complement fixation test (CFT) assays

Although 'old' technology, CFT has been adopted to automation and currently used to test for antibodies to *C. burnetii* phase 2 antigens, by an automated format. This is not widely used and lacks sensitivity but is a useful confirmation test if a screening test (e.g. EIA) is positive. It should not be used as a screening test done to low sensitivity.

Other serology assays

Other serological assays are reported in the literature, such as latex agglutination (de la Fuente 1989), but will not be discussed further as they are not currently used in Australia.

B. The use of NAA for diagnosing rickettsial / Q-fever infection

The detection of nucleic acid (usually DNA) characteristic of a particular microbe is a recent development in the routine diagnostic laboratory (La Scola and Raoult 1997; Leitner 2002; Furuya 1993; Zhang 1998; Fenollar 2004). Many commercial kits are now available but none yet for rickettsial/Q-fever diagnosis. This is a pity because the detection of specific microbial DNA (usually in blood, but other specimen types may be used e.g. urine for *Chlamydia trachomatis*) is a very sensitive and very specific test for detecting a microbe. It does not give information on whether the microbe is dead or alive, but during an acute infection, with the patient still unwell, it is most likely that a positive NAA test is equivalent to detection of a 'viable microbe'.

NAA assays can sometimes be quantified (Jiang 2004), so a 'microbial load' or concentration in the patient sample being tested can be determined e.g. viral load of Hepatitis C virus, for monitoring response to therapy.

NAA tests for rickettsial/Q-fever tend to be in-house assays developed by 'reference laboratories' (Fournier & Raoult, 2004).

They have the very powerful advantage of being positive at a time in the evolution of the patients' illness when serology is usually negative. They are positive early in the illness, often while the patient is still symptomatic.

They are not widely available which is a greater disadvantage. Only a few laboratories in Australia run these assays as a regular diagnostic service and they are often long distances from the patient and the treating doctor.

Assays are 'home grown' and based on the amplification of genes (or parts of genes) that are specific for only that microbe, but present in all variants of that microbe. For example, the citrate synthase gene and the 17 k Da antigen gene are present in all known *Rickettsia* spp, so a NAA assay based on these genes should diagnose all *Rickettsia* spp infections.

However, the omp A gene is present in only the spotted fever group (SFG) rickettsia. So an assay based on this gene will detect a case of Flinders Island spotted fever (*R. honei*), a SFG infection, but not murine typhus (*R. typhi*), a typhus group (TG) infection. Similarly, in Q-fever, a NAA assay based on the Com I gene (coding for a surface, outer-envelope antigen) will detect all cases, but an assay based on IS IIIIa will not, as there appears to be at least one strain without this insertion sequence.

Thus, specificity of the assay depends on the genes selected and consequently the primers used to amplify the gene.

Sensitivity is nearly always good in NAA assays (as amplification of the gene enhances the detection of the microbe). Nevertheless, the sensitivity of assays can vary and an assay detecting a gene with multiple copies (e.g. IS IIIIa) will be more sensitive than an assay detecting a gene which has only one copy per genome (e.g. Com I).

Sensitivity of a NAA test can be compromised by various technical factors e.g. too much human DNA; failure of the polymerase to work; failure to extract the microbial DNA, etc. causing false-negative results, as can the specificity (usually due to contamination of the assay mixture with extraneous

DNA, not part of the original specimen, causing false-positive results).

Fortunately the problem of DNA contamination is greatly reduced by the use of real-time PCR assays, where the reaction tube is not opened and the amplified DNA is detected in real time by fluorescent markers measured electronically. This type of assay is now replacing gel-based PCR in the diagnostic laboratory. It is extremely sensitive, highly specific and has a rapid turn-around-time, making it ideal for the routine diagnostic laboratory or reference laboratory.

Real time PCR assay for *Rickettsia* spp. has recently been developed and is in routine use at the ARRL, Geelong (Stenos , in press).

C. The use of culture for diagnosing rickettsia/Q-fever infection

While culture is the time-honoured method for diagnosing bacterial infections, it is not suitable as a routine for Rickettsia spp, Orientia spp and Coxiella spp except in specialised laboratories (Musso and Raoult 1995; Marrero and Raoult 1989; Birg 1999).

Being obligate intracellular bacteria, tissue culture must be used to grow rickettsia and there are a number of technical difficulties, not least of which is biocontainment. As culture is not a routine diagnostic test for rickettsia, rather a research tool, it will not be discussed further.

D. The use of direct staining for diagnosing rickettsia/Q-fever infection

The direct detection of these bacteria in patient specimens is rarely done in Australia. However, it is possible to stain biopsies of eschars or rashes with either non-specific stains such as the Gimemez stain (Gimemez 1964) where the rickettsiae stain red and the background tissue green or specific stains using specific antisera, where detection involves immunofluorescence or enzyme immunohistology (Raoult 1984; Walker 1989). Rickettsia may be seen within endothelial cells and coxiella within monocyte/macrophage cells. Coxiella occur within a phagolysosomal vacuole, whereas rickettsiae are free in the host cell cytoplasm or nucleus. Even patient's peripheral leucocytes may be stained to show rickettsiae (Walsh 2001).

Conclusion

Clinical and laboratory diagnosis of rickettsial disease is challenging. The clinician must first think of the diagnosis and the laboratory must then confirm or refute it – a partnership which, when optimal, greatly assists in the recovery of the patient by ensuring prompt, appropriate antibiotic therapy.

Serology is the mainstay of laboratory diagnosis, with micro-immunofluorescence the favoured modality but NAA is likely to become more important in the future, especially during acute illness.

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