The Case for Valid Rickettsia IgM Assays

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Abstract

A 2014 paper in the American Journal of Tropical Medicine concluded "Use of IgM antibodies should be reconsidered as a basis for diagnosis and public health reporting of RMSF and other spotted fever group rickettsiae in the United States." We suggest this warning be confined only to assays using whole-cell Rickettsia antigens, the IgM assay on which this conclusion was based, and to extend this to include typhus group rickettsiosis. Such warnings should never to taken to mean that properly designed IgM assays should not be utilized in confirming acute rickettsiosis. The assumption has too often been that IFA or MIF are the only assays available, although both western immunoblot and ELISA assays have been shown to be both accurate and sensitive. Comparative results of MIF and ELISA performed in our laboratory demonstrate that the removal of LPS from the immunodominant protein antigens (native rOmp A and/or rOmpB) produces spotted fever and typhus group IgM ELISA assays that are both sensitive and specific. As removal of LPS from whole cell antigens is not realistic due to the crystalline nature of the s-layer, whole cell antigens should not be utilized for IgM antibody assays due to unacceptably high false-positive rates. Attempts were made to adsorb anti-LPS reactivity in serum samples using LPS-coated microbeads (SFG or TG-specific) as a pre-treatment step, but the decrease in false-positive titers by MIF was less than a single two-fold dilution. Similar results were found using Weil-Felix antigens for adsorption.

Introduction

In many clinical laboratories both domestic and internationally continue to use whole cell IFA and MIF assays for Rickettsia serology. It remains a wide-spread fact that IFA is still considered the "Gold Standard" assay, although this is only true for IgG testing. The chief drawback of whole cell antigen is the lipopolysaccharide (LPS) antigen which is the main false-target, producing false-positives due to cross reactivity of IgM with enterobacterial LPS. This natural immunity is sufficiently strong as to preclude serum absorption techniques and LPS cannot be selectively removed from the crystalline s-layer of the intact whole cell Rickettsia antigen.

Reagents

For MIF testing, our Fuller Laboratories 2-antigen MIF slides (R20-12) were utilized according to assay kit protocols. These slides contain separate dots of Rickettsia rickettsii and R. typhi in each well within a background matrix. In short, the sera were pre-treated with high-titer goat anti-human IgG (γ chain-specific) to precipitate IgG-class antibody and, after allowing 10-20 minutes for this reaction, further diluted in PBS to 1:64. Treated serum dilutions were incubated in slide wells for 30 minutes, washed with PBS and treated with Alexafluor 488-conjugated goat anti-human IgM (5μ-specific) for 30 minutes.

For ELISA IgM testing, our Fuller Laboratories protocols for spotted fever (RRM-96K) and typhus group (RTM-96K) were used as described in the kit inserts. In short, serum specimens were pre-treated with goat anti-human IgG (γ chain-specific) to precipitate IgG-class antibody and, after allowing 10-20 minutes for this reaction, further diluted in PBS to 1:100 in a diluent containing Tween 20 and bovine serum albumin. These pre-treated sera were incubated in test wells at ambient temperature (23˚C) for 60 minutes, then washed 3X with PBS-Tween 20 washer buffer. HRP-conjugated goat anti-human IgM (5μ-specific) was added for another 60 minutes at ambient temperature, followed by 3 wash steps using PBS-Tween 20. A TMB substrate was reacted with test wells for 10 minutes before adding the sulfuric acid STOP Solution. Test and Control wells were quantitated at 450 nm and absorbance values compared with Cutoff Calibrator values. Values higher than the Cutoff Calibrators (± 10%) are positive results.

Serum samples (n = 100) from normal healthy donors were supplied by Equitech-Bio, Inc (Kerrville, TX) and 4 were used for other controls, leaving n = 96.

Results

Spotted fever IgM

By MIF a total of 43 sera were at least 1+ positive (4-point scale) with 13 of those sera at a 2+ or higher reading. With a sensitive assay like this, perhaps screening at 1:128 for IgM would be more meaningful, dropping the false-positive rate from 44.8% to 13.5%.

	MIF	ELISA
Positive	43	1
Negative	53	95

Typhus group IgM

By MIF a total of 19 sera were at least 1+ positive (4-point scale) with 13 of those sera at a 2+ or higher reading. As with the spotted fever, perhaps screening at 1:128 for IgM by MIF would be more meaningful, dropping the false-positive rate from 19.8% to 13.5%.

	MIF	ELISA
Positive	19	1
Negative	81	95

Note: A single serum was found ELISA positive on both assays (#84) and has been used as a Positive Control.

RRM-96K	R20M-SFG	R20M-TG	RTM-96K
Neg	Pos	Pos	Neg
Neg	Pos	Pos	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	±	±	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Pos	Neg
Neg	±	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	±	Neg
Neg	±	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	±	Neg	Neg
Neg	Pos	Pos	Neg
Neg	Pos	Pos	Neg
Neg	Neg	Neg	Neg
Neg	±	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Pos	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	±	Neg	Neg
Neg	Pos	±	±
Neg	Pos	±	Neg
Neg	Neg	Neg	Neg
Neg	±	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	±	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg

RRM-96K	R20M-SFG	R20M-TG	RTM-96K
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	±	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Pos	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Pos	Neg
Neg	Neg	Pos	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Pos	±	Neg
Neg	±	Pos	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Pos	Neg
Neg	Pos	Neg	Neg
Neg	Pos	±	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Pos	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Pos	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Neg	Neg	±
Neg	Neg	Neg	Neg
Neg	Neg	Neg	Neg
Pos	Pos	Pos	Pos
Neg	Pos	Pos	Neg
Neg	Pos	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Neg	Pos	Neg
Neg	±	Neg	Neg
Neg	Neg	Neg	Neg
Neg	±	±	Neg
Neg	Pos	Pos	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	Neg	Neg
Neg	Neg	Neg	Neg
Neg	Pos	±	Neg
Neg	Pos	Pos	Neg
Neg	±	Pos	Neg

Discussion

The ELISA assays used in this comparison have been commercially available since 2009 and we have supplied reagents for numerous clinical validations around the world. Our original Internal validations compared results with a qualitative Western Immunoblot to set the Cutoff Calibrator. The coating antigens for these assays are native Outer Membrane Proteins (rOmp), purified while specifically removing any trace of the lipopolysaccharide (LPS) antigens found in the rickettsial s-layer. The spotted fever assay (RRM-96K) utilizes the Omp A + Omp B heteroduplex from Rickettsia rickettsii, although the range of reactivity across the major pathogenic clades is rather broad due to the antibody class. The assay for typhus group utilizes the purified Omp B from Rickettsia typhi, which also detects reactivity to R. prowazekii.

Cross reactivity of IgM class antibody to enterobacterial LPS is often mentioned in discussing false-positive results in Rickettsia assays and it is most often mentioned in relegating all IgM assays to the "clinically unreliable" category. This information regarding Rickettsia IgM assays is not common knowledge among clinicians or clinical laboratory personnel, who are generally unaware of the types of assays that have been developed to generate the high sensitivity and specificity required.

A series of remedial assays were attempted to remove anti-LPS reactivity from clinical sera, including pre-incubations with liquid-phase LPS, solid-phase LPS on micro-beads and Weil-Felix antigens. The lack of meaningful decreases in titer point to an excessive expense involved. The preferred method in this case is to simply remove the LPS antigen from the test assay and use only the immunodominant protein antigens.