

Technical report

An immunoblotting procedure comprising O = 9,12 and H = d antigens as an alternative to the Widal agglutination assay

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Abstract

Aims—To compare the established Widal agglutination assay with an immunoblotting procedure.

Methods—110 sera were used to compare the established Widal agglutination assay with an immunoblotting procedure incorporating lipopolysaccharide (LPS) (O = 9,12) and flagellar (H = d) antigens. **Results**—Antibodies to the LPS antigens were detected in 18 sera by the Widal assay and in 37 by immunoblotting. Antibodies to the flagellar antigens were detected in 27 sera by Widal assay and in 25 by immunoblotting.

Conclusions—An immunoblotting procedure incorporating O = 9,12 LPS and H = d flagellar antigens was rapid and more sensitive than the established Widal agglutination assay for providing evidence of infection with *S typhi*.

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Keywords: immunoblotting; Widal reaction; *S typhi*

In the United Kingdom, most cases of typhoid occur in travellers returning from areas where this disease is endemic. The diagnosis of typhoid can be confirmed by culturing *Salmonella typhi* from blood or faeces; however, in the absence of culturable *S typhi*, serological methods offer an alternative approach to provide evidence of infection. Patients infected with *S typhi* have been known to produce serum antibodies to this organism since the early studies of Widal,¹ performed over a century ago. The Widal test was based on the ability of patients' antibodies to agglutinate *S typhi* bacteria, and provided a valuable adjunct to established bacteriology. Variation in results obtained by different laboratories² initiated attempts to standardise the Widal agglutination assay,³ and with the use of Dreyer's "standard" bacterial suspensions, a definitive Widal assay was issued by the Medical Research Council (MRC) Standards Laboratory at Oxford.

The antigenic code for *S typhi* has been designated as [Vi] 9,12:d by the Kauffmann-White typing scheme.⁴ The MRC protocol emphasised the importance of using standard

antigen preparations and specific control sera, and the significance of adhering to consistent experimental procedures³; however, even with these specific regimens, variation in serological reproducibility was encountered.³ With the passage of time, the Widal assay has been modified further by various workers; for example, in certain studies the Widal agglutination assay has been performed on glass slides and not in the recommended plates with round bottomed wells.³ Also, antibody titres considered to indicate significant levels of serum antibodies specific for O = 9,12 lipopolysaccharide antigens are no longer standardised and can vary from 1/20⁵ to 1/40,⁶ 1/80,⁸ or 1/160.⁹ To improve the serodiagnosis of typhoid, more modern immunoassays have been considered as a replacement for the Widal assay. These include enzyme linked immunosorbent assays (ELISA) and crossed immunoelectrophoresis.¹⁰⁻¹²

Studies from this laboratory showed that SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) combined with immunoblotting was considerably more sensitive for detecting human antibodies to the lipopolysaccharide (LPS) and flagellar antigens of *S typhi* than the Widal assay.¹³ Also, immunoblotting was shown to have the advantage over an ELISA by requiring only partially purified flagellar antigens. Subsequent studies with sera from patients who were culture positive for *S typhi* showed that immunoblotting detected antibodies to the O = 9,12 LPS antigens in 99% of sera.¹⁴

The Laboratory of Enteric Pathogens (LEP) is the United Kingdom reference centre for typhoid serology and screens sera for antibodies to *S typhi* in clinical cases, carriers, and healthy persons. In the present study, 110 sera received by the LEP for routine typhoid serology were used to establish whether SDS-PAGE/immunoblotting would be a suitable test to replace the established Widal agglutination assay.

Methods

BACTERIA

Salmonella enteritidis strain P132344 (O = 1,9,12; H = g,m) and *S meunchen* strain JT54 (O = 6,8; H = d) were used to prepare lipopolysaccharide and flagella antigens.¹⁵

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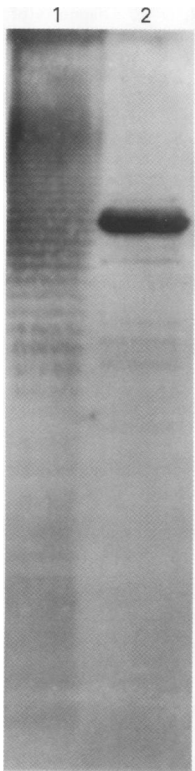


Figure 1 Thirty seven of the 110 sera contained antibodies to the O = 9,12 LPS, giving a strong reaction by immunoblot (lane 1); 25 sera contained antibodies to the 65 kDa H = d flagellar protein subunits (lane 2).

Table 1 Comparison of immunoblotting with the Widal agglutination test when screening 110 sera for antibodies to lipopolysaccharide (LPS) antigens (O=9,12) and flagellar antigens (H=d)

	Immunoblotting		Widal agglutination	
	LPS	Flagellar	LPS	Flagellar
No of positive sera	37	25	18	27

SERA

Overall 110 sera had been referred to the Laboratory of Enteric Pathogens for routine Widal serology. Sera were predominantly from patients suspected of having been infected with *S typhi*, but also included six sera from patients with reactive arthritis, five apparently healthy persons involved with catering or water industries, and three people vaccinated against typhoid. All sera were stored at -30°C until used.

LIPOPOLYSACCHARIDE/FLAGELLA

LPS was prepared from *S enteritidis* using proteinase-K digestion (Sigma) and hot phenol extraction of bacterial outer membranes.¹³ Flagella were partially purified from *S muenchen* by heat extraction (60°C , 30 minutes).¹⁴ Aliquots of preparations of LPS and flagella were stored at -30°C until required.

SDS-PAGE/IMMUNOBLOTTING

SDS-PAGE and immunoblotting was performed using a mini gel system.¹⁵ One microgram of LPS or 10 μg flagellar protein were loaded per lane of a gel comprising a 4.5% stacking gel and a 12.5% separation gel. Following electrophoresis, replicate profiles of LPS and flagellar protein were either stained¹⁶ for assessment of purity and profile resolution, or used for immunoblotting and reaction with human sera. For immunoblotting, profiles were transferred onto nitrocellulose membranes and reacted with sera (30 μl per lane), and bound antibodies detected with a goat antihuman polyvalent antibody conjugated with alkaline phosphatase (Sigma). Antibody-antibody-conjugate complexes were detected with an enzyme substrate comprising nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma).¹⁵ Immunoblotting reactions were graded between 0 and 3, with 0 indicating a complete absence of antibody binding and 3 where antibody binding was readily observed. Only sera giving an immunoblot reaction of 2 or 3 was considered antibody positive for this study.

WIDAL SEROLOGY

Widal serology was performed based on the standard procedure described by Felix and Gardner.² In the LEP an agglutinating antibody titre of $> 1:100$ is considered as significant for antibodies to LPS and flagellar antigens.¹³

Results

Of the 110 sera examined, 42 did not have antibodies to either LPS or flagellar antigens, by either of the methods under investigation. Antibodies to LPS and flagellar antigens were

detected by immunoblotting in 37 and 25 sera, respectively (table 1; fig 1). Eleven sera contained antibodies to both LPS and flagella antigens, 26 had antibodies to LPS only, and 14 had antibodies to flagella only.

The Widal agglutination assay detected 18 and 27 sera with antibodies to LPS and flagella antigens, respectively (table 1). Four sera had antibodies to both LPS and flagellar antigens, 16 had antibodies to LPS only, and 23 had antibodies to flagella only.

The five sera from water workers were shown not to contain antibodies to LPS or flagella antigens by both tests. The three sera from people who had been vaccinated against typhoid were found to contain antibodies to LPS (2) and flagellar antigens (3) by immunoblotting, but the Widal assay detected only anti-flagellar antibodies in two of these sera. Sera with high levels of antibodies by Widal test but antibody negative by immunoblotting were not detected.

Discussion

In this study we used 110 sera submitted to the LEP for routine Widal serology, to compare the established Widal agglutination assay with an immunoblotting procedure incorporating LPS (O = 9,12) and partially purified flagellar proteins (H = d). The immunoblot procedure detected over twice as many sera with antibodies to O = 9,12 LPS as compared with the Widal agglutination assay. Both procedures detected a similar number of sera with antibodies to flagellar antigens. The Widal agglutination assay detected four sera with antibodies to both LPS and flagellar antigens; however, immunoblotting detected 11 patients with serum antibodies to both of these antigens.

Antibodies to the O = 9,12 LPS only were detected in 16 and 26 sera by the Widal and immunoblot assays, respectively. It has been shown that patients infected with *S enteritidis* also produce antibodies to the 9,12 LPS antigens^{17,18}; therefore, detecting antibodies to LPS alone would not distinguish patients infected with *S typhi* from those infected with *S enteritidis*. Also, studies from this laboratory have shown that approximately one third of patients known to have been infected with *S typhi* may not produce antibodies to flagella antigens during infection.¹⁴ These facts emphasise the importance of considering patients' symptoms, any history of foreign travel, and records of previous typhoid vaccinations when interpreting the results of serological tests.

From this study we conclude that an immunoblotting procedure incorporating O = 9,12 LPS and H = d flagellar antigens is more sensitive than the established Widal agglutination assay for providing evidence of infection with *S typhi*. Although the immunoblot test requires slightly more elaborate laboratory equipment than the Widal agglutination assay, the apparatus required for SDS-PAGE and immunoblotting has been miniaturised extensively and simplified considerably. The immunoblot can be performed in a single working day, in contrast to the Widal agglutination assay which

takes two days. We suggest that the immunoblot we describe should be considered as a viable alternative to the long established Widal agglutination assay.

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