Serodiagnosis of *Salmonella enterica* serovar Typhi and *S. enterica* serovars Paratyphi A, B and C human infections

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The aim of this study was to evaluate an immunoassay for the detection of human serum antibodies to the LPS and flagellar antigens of *Salmonella* Typhi and *Salmonella* Paratyphi A, B and C, and to the Vi capsular polysaccharide of *S. Typhi* and *S. Paratyphi* C. A total of 330 sera were used; these originated from 15 patients who were culture-positive for *S. Typhi* and 15 healthy controls, together with 300 sera submitted to the Laboratory of Enteric Pathogens for *Salmonella* serodiagnosis. By SDS-PAGE/immunoblotting, all 15 sera from culture-positive patients had serum antibodies to the 9,12 LPS antigens and 10 had antibodies to the ‘d’ flagellar antigens. Of the 300 reference sera, 22 had antibodies to the 9,12 LPS antigens, one to the 1,4,5,12 LPS antigens and 12 to the 6,7 LPS antigens. Only two sera had antibodies to flagellar antigens, one of which bound to the ‘b’ and the other to the ‘d’ antigen. An ELISA was developed that successfully detected serum antibodies to the Vi capsular polysaccharides, but because of the kinetics of serum antibody production to the Vi, these antibodies may be of limited value in the serodiagnosis of acute infection with *S. Typhi* and *S. Paratyphi* C. The immunoassays described here provide a sensitive means of detecting serum antibodies to the LPS, flagellar and Vi antigens of *S. Typhi* and *S. Paratyphi*, and constitute a viable replacement for the Widal assay for the screening of sera. The *Salmonella* serodiagnosis protocols described here are the new standard operating procedures used by the Health Protection Agency's National Salmonella Reference Centre based in the Laboratory of Enteric Pathogens, Colindale, UK.

INTRODUCTION

Typhoid remains a serious health problem in many regions of the world, with some 16 million cases and 600,000 deaths occurring annually (Parry, 2004). The major causes of typhoid fever are *Salmonella enterica* serovar Typhi (*S. Typhi*) and also, to a lesser extent, strains of *S. enterica* belonging to serovars Paratyphi (*S. Paratyphi*) A, B and C. In the UK, cases of typhoid tend to occur in travellers returning from locations where this disease is endemic or in people who have been in contact with patients who have become asymptomatic carriers of *S. Typhi* or *S. Paratyphi* (Anonymous, 1961). In order to facilitate the treatment of patients infected with these bacteria and to study the epidemiology of infections and identify patients who become asymptomatic carriers of these organisms, it is important to provide evidence of infection. The isolation of *S. Typhi* or *S. Paratyphi* from the blood or faeces of patients confirms diagnosis; however, in the absence of a culturable organism, patients' antibodies may provide evidence of infection with these serovars of *Salmonella*. As the National Reference Centre for *Salmonella*, the Laboratory of Enteric Pathogens (LEP) receives approximately 120 sera per year for serodiagnosis and until recently this was performed using the established Widal assay (Felix, 1950). The four key serovars causing typhoid express distinct surface antigens and these form the basis of serological tests. The antigenic structure for *S. Typhi* has been designated 9,12: d; Vi denoting the 9,12 LPS antigens, the ‘d’ flagellar antigens and the ‘Vi’ antigens. The abbreviation ‘Vi’ refers to the virulence-associated capsular polysaccharide expressed by strains of *S. Typhi* and *S.
Paratyphi C (Felix et al., 1935). Similarly, strains of S. Paratyphi have the antigenic structures 1,2,12: a (S. Paratyphi A), 1,4,5,12: b (S. Paratyphi B) and 6,7: c; Vi (S. Paratyphi C) (Rowe & Hall, 1989). In 1896, Widal reported that sera from patients with clinical typhoid 'clumped' the bacteria isolated from the host (Widal, 1896) and this agglutination method formed the basis of serodiagnostic tests for typhoid for over 100 years. Traditionally, the Widal assay involved an agglutination assay using formalin-fixed bacteria expressing the LPS and flagellar antigens, with antibodies to the Vi capsule being detected using a strain of S. Typhi expressing a Vi capsule but lacking LPS and flagella antigens (Bhatnagar, 1938; Bhatnagar et al., 1938; Felix & Pitt, 1951). The Widal agglutination assays were refined and standardized in 1954 by Felix & Bensted (1954), who advocated the use of specific type strains expressing the salient antigens in a highly specific agglutination assay using Dreyer's tubes (Anonymous, 1961). Over the following years, the assay was modified in both the test and the interpretation of the results. For example, antibody titres considered to be clinically relevant against the 9, 12 LPS antigens were set at values ranging from 1:20 (Hoffman et al., 1986), 1:40 (Levine et al., 1978) and 1:80 (Buck et al., 1987) to 1:160 (Pang & Puthucheary, 1983). With concerns over the non-specificity of Widal agglutination reactions (Reynolds et al., 1970; Zuerlein & Smith, 1985; Koelman et al., 1992) and the interpretation of results (Schroeder, 1968), the agglutination tests became plagued with controversy and doubt was cast on the value of the Widal serodiagnostic assay (Olopoenia & King, 2000).

Serological tests using more up-to-date technology were developed, fuelled by the need for rapid screening of patients suspected of having typhoid; however, some of these tests were reported to give no improvement on the original Widal agglutination test (Dutta et al., 2006). The techniques of SDS-PAGE/immunoblotting proved highly efficient for the detection of human antibodies to S. Typhi antigens (Chart et al., 1995, 1997, 2000), enabling the detection of all classes of serum antibody and not solely agglutinating antibodies. Although not a rapid technique, SDS-PAGE/immunoblotting has the advantage of enabling direct observation of antibody binding to antigen profiles, avoiding problems with non-specific antibody binding.

The aim of the present study was to evaluate a serodiagnostic assay using SDS-PAGE/immunoblotting employing LPS and flagellar antigens of S. Typhi and S. Paratyphi A, B and C, and an ELISA based on purified Vi capsular polysaccharide, using a total of 330 sera. We present a highly specific assay that has replaced the Widal assay for the serodiagnosis of infections with S. Typhi and S. Paratyphi A, B and C in the LEP.

**METHODS**

**Bacteria.** Strains of *Salmonella Enteritidis* (P132344; 1,9,12; g, m), S. Paratyphi A (1,2,12: a), *Salmonella* Typhimurium (P459327; 1,4,5,12: i) and *Salmonella* Livingstone (JT300; 6,7: d) were used for the preparation of LPS antigens. Flagellar antigens were prepared from strains of *Salmonella* Muenchen (JT54; 6,8: d), *Salmonella* Doncaster (JT1293; 6,8: a), *Salmonella* Ohio (JT462; 6,7: b) and *Salmonella* Goeteborg (JT807; 9,12: c). All strains were from the culture collection held by the LEP. For the preparation of LPS, bacteria were grown on blood agar (37°C for 16 h), and for the preparation of flagella, bacteria were passaged through semi-solid agar (Craigie medium) twice prior to culture on blood agar (37°C for 16 h).

**Patients/sera.** Fifteen sera were from culture-positive patients infected with *S. Typhi* and 15 were from apparently healthy controls. Sera from 300 patients had been submitted to the LEP for reference typhoid serology over a 30 month period. The patients comprised 128 females (mean age 38.8 ± 17.6 years, mode 54 years, median 37.5 years) and 154 males (mean age 36.4 ± 17.6 years, mode 26 years, median 33 years). The gender of 18 patients was not known.

Of the 300 patients, 77 had reported a history of recent travel abroad, with 20 having visited India, 15 having visited Pakistan and up to six having visited Africa, Bangladesh, Egypt, China or Mexico. Thirty-eight of the 300 patients had diarrhoea and eight of these also presented with abdominal pain. Thirteen patients had experienced vomiting and 82 had been diagnosed with pyrexia. A serum sample from a patient with clinical typhoid and with antibodies to the Vi capsular polysaccharide was used as a positive control in ELISA assays; this serum was from the serum bank held by the LEP.

**Non-human sera.** Rabbit sera with antibodies to the LPS antigens 1,9,12; 1,2,12; 1,4,5,12; and 6,7 were used to confirm the presence of these antigens on SDS-PAGE LPS profiles. Similarly, rabbit sera with antibodies to flagellar antigens a, b, c and d were used to confirm the presence of these antigens on flagellar protein subunits produced by SDS-PAGE. These rabbit sera were from the collection of rabbit sera forming the National Salmonella Serotyping facility based in the LEP at Colindale, UK. A horse serum prepared against the Vi antigen (Felix & Petrie, 1938) in the 1950s (Felix & Bensted, 1954) was used to monitor the adsorption of purified Vi capsular polysaccharide to ELISA plates.

**LPS.** For SDS-PAGE and immunoblotting, LPS was prepared by digesting whole bacteria with protease K (Chart et al., 1989). Bacteria were placed in pre-weighed Eppendorf tubes and the cells were suspended in SDS-PAGE sample buffer (Laemmli, 1970) to give a concentration of 1 mg in 30 μl prior to incubation at 100°C for 10 min. After cooling, samples were mixed with an equal volume of SDS-PAGE buffer containing 100 μg protease K in 30 μl (product code P6556; Sigma) prior to incubation at 60°C for 1 h.

**Flagella.** For the preparation of flagella, bacteria were cultured in semi-solid agar (Craigie medium) prior to growth on blood agar. Bacteria were examined initially for expression of flagella by electron microscopy and those observed to express flagella were suspended in saline solution prior to flagella extraction. Bacterial suspensions were incubated at 60°C for 1 h to release flagella from the cell surface before the bacteria were sedimented by centrifugation (25 000 g for 60 min). The flagella-rich supernatant was harvested and examined by electron microscopy to ensure the presence of flagella before determination of protein concentration using the Lowry method.

**Electron microscopy.** Bacteria were examined by electron microscopy by taking a sweep of bacteria with a loop and placing the cells in 0.5 ml saline containing 1% (w/v) formalin. Drops of bacterial suspension (~50 μl) were placed on a sheet of Parafilm and overlaid with a Formvar-coated copper electron microscope grid for 1 min, prior to transfer to a drop of 1% (w/v) aqueous ammonium molybdate for 1 min. Excess liquid was removed with tissue paper.
and grids were dried at 37 °C for 10 min. Grids were examined with a Phillips EM420 transmission electron microscope and images were captured with a digital camera.

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed using an Anito mini-gel apparatus (Genetic Research Instruments) using the method of Laemml (1970). Preparations of LPS, representing 80 μg digested cell mass, or flagella (5 μg) were used per lane of gels comprising a 4.5% stacking gel and a 12.5% separation gel. Protein preparations were incubated at 100 °C for 5 min prior to electrophoresis, performed at 50 A for 30 min. LPS profiles were detected with a sensitive silver stain (Tsai & Frasch, 1982) and proteins were detected with Coomassie brilliant blue (Chart et al., 1989), or profiles were transferred to nitrocellulose paper by immunoblotting (0.50 A for 1 h; Chart et al., 1989). Profiles immobilized on sheets of nitrocellulose paper were blocked with 3% skimmed milk in PBS (milk/PBS) for 30 min and reacted with 30 μl human or rabbit serum in 5 ml milk/PBS for 60 min. After washing in PBS/Tween 20 (three times for 10 min each), profiles were reacted with 5 μl per lane of goat anti-human polyvalent Ig conjugated with alkaline phosphatase (product code 075-1007; Insight Biotechnology) in milk/ PBS for 60 min or with 5 μl goat anti-rabbit IgG or IgM conjugated with alkaline phosphatase (product code A3937, Sigma; product code 4020-04, Southern Biotechnology) in milk/PBS for 60 min. After washing, as before, profiles were placed in substrate buffer [0.1 M Tris/HCl (pH 9.8), 0.09 M NaCl, 0.15 M MgCl2.6H2O] containing 45 μl nitro blue tetrazolium (75 mg ml⁻¹ in 70% aqueous dimethyl formamide; product code N-6876, Sigma) and 35 μl 5-bromo-4-chloro-3-indolyl phosphate (disodium salt) (50 mg ml⁻¹ in deionized water; product code B-6149, Sigma). For SDS-PAGE, protein standards (product code 161-0304; Bio-Rad) comprising phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) were used to calculate the molecular size of the flagella protein subunits.

**ELISA.** The ELISA was based on a commercial preparation of S. Typhi capsular polysaccharide (TYPHIM Vi; Sanofi Pasteur MSD; Hessel et al., 1999). A horse serum prepared to the Vi of S. Typhi was used to monitor binding of the capsular polysaccharide to ELISA plates. Horse antibodies were detected using a goat anti-human polyvalent Ig antiserum conjugated to alkaline phosphatase (product code A6063; Sigma). Human sera, diluted 1:500 with PBS, were reacted with duplicate wells coated with 0.1 μg Vi capsular polysaccharide in 100 μl coating buffer (1.39 g Na2CO₃, 1–1, 2.93 g NaHCO₃, 1–1, pH 9.6) and blocked with PBS containing BSA (10 g l⁻¹; product code A-2153, Sigma). Sera were also reacted with a duplicate set of uncoated wells that had been blocked with BSA only. Samples of serum (100 μl per well) were added and antibody binding was detected with the same anti-human antibody conjugate as described earlier for immunoblotting, diluted 1:1000 in PBS (100 μl per well). Detection of antibodies of the IgG or IgM class was carried out using a goat anti-human IgG or IgM, conjugated with alkaline phosphatase (product codes A3150 and A3275; Sigma). Alkaline phosphatase-conjugated antibodies were detected by adding 200 μl diethanolamine buffer (1 M diethanolamine, 2 mM MgCl2, pH 9.6) containing 1 mg 4-nitrophenyl phosphate ml⁻¹ (product code N-4645; Sigma). The resultant colour was quantified by measuring the A405 and the results were recorded when a standard positive control serum attained a value of 1.5±5%.

**RESULTS AND DISCUSSION**

The ‘gold standard’ for identifying the cause of an infection is the isolation and identification of the causative agent of disease. In the absence of a viable bacterium, antibody tests can give evidence of infection provided that suitable immunoassays, based on well-characterized antigens, are used. In the present study, LPS antigens comprising 1,9,12; 1,2,12; 1,4,5,12; and 6,7, and flagellar proteins with antigens a, b, c and d were used to prepare SDS-PAGE profiles for reaction with patients’ antibodies by immunoblotting. As shown in Fig. 1 (lanes 1–4), the tests strains were seen to express long-chain LPS migrating in SDS-PAGE gels with a typical ladder pattern when visualized with a silver stain. For the preparation of flagella, bacteria harvested from blood agar plates and shown to express copious numbers of flagella (Fig. 2) were isolated and their flagella were separated (Fig. 2; inset) and examined by SDS-PAGE and Coomassie blue staining. Protein profiles of flagella prepared from S. Muenchen, S. Doncaster, S. Ohio and S. Goeteborg comprised flagellin subunits of approximately 55 kDa (Fig. 1; lanes 6–9).

To investigate the antigenic properties of these antigens, profiles of the four LPS types were reacted with rabbit antibodies specific for LPS antigens 1,9,12; 1,2,12; 1,4,5,12; 1,2,12; 1,4,5,12; and 6,7 were used to prepare SDS-PAGE profiles prior to silver staining (lanes 1–4). Protein profiles of flagella prepared from S. Muenchen, S. Doncaster, S. Ohio and S. Goeteborg comprised flagellin subunits of approximately 55 kDa (lanes 6–9).

Sera from the 15 culture-positive patients gave a strong immunoblot reaction with 1,9,12 LPS (lane 10) and ten of the sera also contained antibodies binding to the ‘d’ flagellar antigens (lane 11). Lane 5 contained standard proteins of 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa.

**Fig. 1.** LPSs with antigens comprising 1,9,12; 1,2,12; 1,4,5,12; and 6,7 were used to prepare SDS-PAGE profiles prior to silver staining (lanes 1–4). Protein profiles of flagella prepared from S. Muenchen, S. Doncaster, S. Ohio and S. Goeteborg comprised flagellin subunits of approximately 55 kDa (lanes 6–9). Sera from the 15 culture-positive patients gave a strong immunoblot reaction with 1,9,12 LPS (lane 10) and ten of the sera also contained antibodies binding to the ‘d’ flagellar antigens (lane 11). Lane 5 contained standard proteins of 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa.

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The appropriate antigens suitable for the detection of polysaccharide used in this study were shown to contain the preparations of LPS, flagella and Vi capsular antigens contained the appropriate antigens for screening antibodies (data not shown), indicating that the preparations contained the appropriate antigens for screening human sera.

The preparations of LPS, flagella and Vi capsular polysaccharide used in this study were shown to contain the appropriate antigens suitable for the detection of patients’ antibodies to S. Typhi and S. Paratyphi.

The sera from the 15 culture-positive patients gave strong immunoblot reactions with the 1,9,12 LPS antigens (Fig. 1, lane 10), confirming that patients mounted a humoral antibody response to LPS during infection with this organism (Chart et al., 1995, 2000; Parry, 2004; House et al., 2005). Ten (67%) of these sera also contained antibodies that bound to the ‘d’ flagellar antigens (Fig. 1, lane 11), which was comparable to 62% obtained in a previous study (Chart et al., 1997), suggesting that not all patients produce antibodies to flagellar antigens. However, in contrast, Brodie (1977), using an agglutination test, detected antibodies to the flagella of S. Typhi more frequently than to 9,12 LPS during an outbreak of typhoid in Aberdeen in 1964 (Brodie, 1977). The reasons for this are not known but may lie in the differences in tests used in the respective studies.

Sera were also tested for antibodies to the Vi capsular antigens by an ELISA based on the TYPHIM Vi capsular polysaccharide, alongside standard positive- and negative-control sera. For the 15 sera from culture-positive patients (Fig. 3) and for this particular representative experiment, ELISA values were recorded when the positive-control serum (no. 1) attained a mean value of 1.52 ± 0.13, at which point the negative control serum (no. 2) gave a mean value of 0.09 ± 0.003. Sera from patients with typhoid (nos 3–17) gave mean values ranging from 0.09 ± 0.01 to 1.01 ± 0.09. Only one serum gave an A405 value of >2.1 (not shown), with 19 sera having A405 values falling between 0.51 and 1.0 (not shown), supporting the theory that patients with an acute infection with S. Typhi may not express antibodies to the Vi antigen (Levine et al., 1989) but develop them only after several weeks post-infection (Felix et al., 1935; Glauer & Richter, 1952; Huckstep, 1962; Robbins & Robbins, 1984; Tacket et al., 1991). Vi-specific antibodies are detected in asymptomatic carriers of S. Typhi (Anonymous, 1961, 1978; Lanata et al., 1983; Losonsky et al., 1987; Lin et al., 1988; Gupta et al., 2006), again indicating that such antibodies require a longer period to develop. Should this be the case, it suggests that the Vi-based immunoassay may only serve to provide evidence of S. Typhi carrier status and may have little or no role in the serodiagnosis of acute infections, intimating that the patients detected here may in fact have been in the convalescent stages of the infection or were asymptomatic carriers or had been exposed to the Vi antigen through vaccination.

The 15 control sera showed no antibody reactions to any of the test antigens.

Of the 300 reference sera, 22 had antibodies binding to 1,9,12 LPS. The observation that nine of these 22 sera contained antibodies that also bound 1,2,12 and 1,4,5,12 LPS antigens indicated that these nine sera contained antibodies that recognized the ‘12’ antigens only, whilst the remaining 13 sera had antibodies binding to the ‘9’ antigen. This was in contrast to previous studies (Chart et al., 1995) where the ‘12’ antigen, and not the ‘9’ or ‘1’ antigen, was found to be the key antigen recognized by patients with antibodies to S. Typhi LPS. This observation serves to illustrate that antibodies to both the ‘9’ and ‘12’ antigens should be assayed as a minimum criterion for serodiagnosis. Twelve sera had antibodies to the 6, 7 LPS antigens, indicating an infection with S. Paratyphi C. Only one of the 300 reference sera contained antibodies to both LPS and flagella antigens, and these indicated infection with S. Paratyphi B, demonstrating that, for effective serodiagnosis, antibodies to both the LPS and flagellar antigens need to be detected. Of the 22 patients with serum antibodies to 1,9,12 LPS, six had been diagnosed with pyrexia and one had experienced diarrhoea. Six had a history of recent foreign travel. Of the 12 patients with serum antibodies to 6,7 LPS, three had been abroad, but their symptoms were not provided.

The 15 control sera gave A405 ELISA values ranging from 0.07 ± 0.002 to 0.25 ± 0.004 with a mean A405 value of
Salmonella with other serovars of must also be considered in the context of antigens shared in countries where typhoid is endemic. The results of serology patients’ symptoms and any recent history of travel to surgical evidence of infection should be assessed in the light of and one convalescent serum. Once obtained, the serolo-
asera from each patient should be tested: one acute serum

improved by testing sera taken 1 week apart (House et al., 1962). To maximize the likelihood of detecting patients’ antibodies to Salmonella antigens, we advocate that two sera from each patient should be tested: one acute serum and one convalescent serum. Once obtained, the serological evidence of infection should be assessed in the light of patients’ symptoms and any recent history of travel to countries where typhoid is endemic. The results of serology must also be considered in the context of antigens shared with other serovars of Salmonella. For example, strains of Salmonella Dublin express the 1,9,12 LPS antigens, express a Vi capsular antigen and may cause typhoid-like illness; however, the LEP receives some 20 isolates of S. Dublin per year (unpublished) and these tend not to be associated with foreign travel. The Salmonella serodiagnosis protocols described here are the new standard operating procedures used by the Health Protection Agency’s National Salmonella Reference Centre based in the LEP. Although serology can only remain an adjunct to bacteriology, the results of serodiagnosis, when considered in the context of patients’ symptoms, can make a valuable addition to the diagnosis of typhoid and paratyphoid fevers and the identification of typhoid carriers.

The immunoassays described here provide a sensitive means of detecting serum antibodies, of all antibody classes, to the LPS and flagellar antigens of S. Typhi and S. Paratyphi, and to the Vi capsular polysaccharide; however, very few of the 300 reference sera were found to contain antibodies to the panel of test antigens. It was concluded that this outcome was probably either due to the patients not having had typhoid or due to the sampling strategy used for taking samples of blood from patients as opposed to the methods used for detecting serum antibodies to key salmonella antigens. It has been demonstrated that the results of serodiagnosis for cases of typhoid fever were improved by testing sera taken 1 week apart (House et al., 2005), and historically it has been standard practice to use acute and convalescent sera for serodiagnosis (Huckstep, 1962). To maximize the likelihood of detecting patients’ antibodies to Salmonella antigens, we advocate that two sera from each patient should be tested: one acute serum and one convalescent serum. Once obtained, the serological evidence of infection should be assessed in the light of patients’ symptoms and any recent history of travel to countries where typhoid is endemic. The results of serology must also be considered in the context of antigens shared with other serovars of Salmonella. For example, strains of Salmonella Dublin express the 1,9,12 LPS antigens, express a Vi capsular antigen and may cause typhoid-like illness; however, the LEP receives some 20 isolates of S. Dublin per year (unpublished) and these tend not to be associated with foreign travel. The Salmonella serodiagnosis protocols described here are the new standard operating procedures used by the Health Protection Agency’s National Salmonella Reference Centre based in the LEP. Although serology can only remain an adjunct to bacteriology, the results of serodiagnosis, when considered in the context of patients’ symptoms, can make a valuable addition to the diagnosis of typhoid and paratyphoid fevers and the identification of typhoid carriers.

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