Polish Journal of Microbiology
2012, Vol. 61, No 2, 137–142

ORGINAL PAPER

Modifications Influencing Widal Test Reactivity in a Novel Microplate Assay

ADEL ALMOGREN[1], ZAHID SHAKOOR[1]*, MUSTAFA HUSSEIN ADAM[1], MOHAMMAD OSMAN GADELRAB[1] and HASSAN ABDULAZIZ MUSA[2]

[1]Department of Pathology, King Khalid University Hospital, King Saud University, Kingdom of Saudi Arabia
[2]Faculty of Medicine, National Rabat University, Khartoum, Sudan

Received 2 November 2011, revised 5 April 2012, accepted 9 April 2012

Abstract

Reliability of the Widal tube agglutination test has been the subject of many controversies over the years. This study was performed to assess the effect of certain modifications on the performance of Widal test in a novel microplate assay. Sera from 37 patients (21 males; 16 females) (mean age 28 ± 7 years) were tested in the Immunology Unit at King Khalid University Hospital, Riyadh. Among them were 26 patients with suspected typhoid fever and 11 had bacteriologically confirmed diagnosis of *Salmonella* infection. The modifications included either the use of 0.5% bovine serum albumin (BSA), absorption of sera with sheep red blood cells (SRBC) or heat inactivation of sera. Compared with Widal tube agglutination test, microplate assay with SRBC absorption of the sera from patients with suspected typhoid fever was not only associated with enhancement of detection titers for both H ($p \leq 0.001$) and O ($p \leq 0.005$) *Salmonella* agglutinins but also the percentage of reactivity. The presence of BSA augmented detection titers for *Salmonella* H agglutinins ($p \leq 0.02$) only. Heat inactivation of sera however was found to be associated with reduction in the detectable titers for both H ($p \leq 0.03$) and O ($p \leq 0.01$) agglutinins. Increased titers of *Salmonella* agglutinins were also evident in 11 patients with confirmed diagnosis of *Salmonella* infection. The novel microplate agglutination assay using the SRBC absorption was associated with enhancement in Widal test reactivity and appears to be a useful alternative for the diagnosis of *Salmonella* infection.

Key words: *Salmonella typhi*, microplate agglutination assay, *Salmonella* agglutinins, Widal tube agglutination test

Introduction

The diagnosis of typhoid fever is usually made by detection of *Salmonella typhi* organisms in blood that is less sensitive than isolation of the organism from bone marrow which is considered as the gold standard (Farooqui *et al.*, 1991). Bone marrow aspiration is an invasive procedure and requires skilled laboratory personnel often not available at primary healthcare facilities (Wain *et al.*, 2008). Similarly in the developing countries where typhoid fever is endemic a relatively small number of laboratories at primary healthcare centres are equiped with the culture facilities requried for isolation of *Salmonella* organisms and the diagnosis of typhoid fever only on clinical evidence remains a problem (Parry *et al.*, 1999; Pang *et al.*, 1983; Choo *et al.*, 1993; Saha *et al.*, 1996). Detection of circulating antibodies against *Salmonella typhi* in serum is therefore useful in the diagnosis of typhoid fever. Rising titers over time or a single high titer of *Salmonella* agglutinnins have been regarded as diagnostically significant (Wain *et al.*, 2008). However, there are several factors which tend to obscure the serological picture; the most important is the sharing of antigens that stimulate antibody production by a large number of organisms from the same genus or from other related organisms yelding false positive results (Jindal *et al.*, 1992; Chart *et al.*, 1994a; Chart *et al.*, 1994b).

A number of attempts have been made in the past to develop a reliable serological technique for the diagnosis of typhoid fever (Duthie and French, 1990). These include the Widal agglutination test, haemagglutination test, enzyme-linked immunosorbent assay, immunoelectrophoresis and polymerase chain reaction test (Rai *et al.*, 1989; Barrett *et al.*, 1983; Nardiello *et al.*, 1984; Verdugo-Rodriguez *et al.*, 1993; Jesudason *et al.*, 1998; Chart *et al.*, 1997; Sharma *et al.*, 1997). None of these tests could be adopted for routine use on account of various reasons whereby the Widal tube agglutination test remained a simple, inexpensive, semiquantitative

* Corresponding author: Z. Shakoor, Department of Pathology, King Khalid University Hospital, King Saud University, Riyadh 11461, Kingdom of Saudi Arabia; phone: 00966-1-4671299; fax: 00966-1-4671842; **e-mail: shakoor_zahid@yahoo.com**

agglutination test for detecting *Salmonella* agglutinins (Parry *et al.*, 1999; Choo *et al.*, 1993; Saha *et al.*, 1996; Coovadia *et al.*, 1986).

Despite being used most frequently the specificity and sensitivity of the Widal test has been debated for many years, especially in the endemic areas where typhoid fever co-exists with non-typhoid febrile illnesses such as malaria and tuberculosis (Rai *et al.*, 1989; Ghosh *et al.*, 2001; Bakr *et al.*, 2011; Keddy *et al.*, 2011). Because of its limited diagnostic capability (Omuse *et al.*, 2010) several modifications of the Widal test such as the slide agglutination test, (Hoffman *et al.*, 1986) plate micro-agglutination test (Pang *et al.*, 1983; Clegg *et al.*, 1994; Barsoum and Awad, 1972), use of 2-mercaptoethanol for the detection of IgM (Jindal *et al.*, 1992) and *Salmonella para* A, *para* B and *Escherichia coli* absorption (Rai *et al.*, 1989) have been investigated but none of these could gain a reasonable acceptance. The need for a tangible test for the diagnosis of *Salmonella* infection therefore remains (Baker *et al.*, 2010) and this study examines the effect of certain modifications on the performance of Widal test.

Experimental

Materials and Methods

Study population. A total of 37 patients including 21 males and 16 females with the mean age of 28 ± 7 years were included in the study. This group of patients comprised of 26 patients with a febrile illness with clinical suspicion of typhoid fever where the treating physician did not request bacterial cultures for isolation of the *Salmonella* organism. The remaining 11 patients had a definitive diagnosis of typhoid fever based on the isolation of *Salmonella* organisms either from blood or stool specimens. The diagnostic titers for H and O agglutinins were either equal to or greater than 1:160 and 1:80, respectively. Means were compared using Student **t** test and $p \leq 0.05$ was considered significant.

Blood sample collection. 3 ml of venous blood was collected by venipuncture and serum was obtained by allowing the blood to clot at the room temperature. A 0.5 ml aliquot was used for Widal tube agglutination and the rest of the serum sample was stored at –20°C until use.

Determination of optimal concentration of BSA. The optimal concentration of BSA was determined by checkerboard titration. Various concentrations of BSA in normal saline ranging from 0.1% to 5% were tested and 0.5% BSA in normal saline was shown to perform best in terms of interpretation of the results and reproducibility.

SRBC absorption. Commercially prepared 10% non-sensitized sheep erythrocytes (Carter Wallace Inc, USA) were used for absorption. To 190 µl of absorbent cells in a test tube 10 µl of undiluted serum sample was added to obtain a serum dilution of 1:20. The contents were mixed gently using a microplate shaker and incubated at room temperature for one hour and then centrifuged at 3000 rpm for 10 minutes. Supernatant was collected and used for microplate agglutination assay.

Heat inactivation of sera. Serum samples were placed in a water bath at 56°C for 30 minutes and then cooled immediately in running tap water prior to be used in the microplate agglutination assay.

Microplate agglutination assay. 50 µl of 0.5% BSA in normal saline was dispensed in all the wells of a U-type micro-titer plate. In the first well of each row 50 µl of serum sample was added and double dilutions were made along the rows up to a final dilution of 1:20480. Aliquots of 50 µL each from 1:100 diluted homogenous *Salmonella typhi* suspensions (Murex, UK) for H and O antigens were then added to the respective wells. The microplate was sealed with a plastic cover, the contents were mixed by gentle shaking and this was followed by incubation at 37°C for 18 hours. After incubation the seal was removed from the plate and the agglutination was read on a Microtitre mirror. A positive reaction was indicated by a smooth or irregular mat shape settlement at the bottom of each well in the microtitre plate, whereas the settling of antigen to a button shape was interpreted as a negative reaction. The titer was determined as the last dilution of serum sample showing a mat formation.

Widal tube agglutination test. The standard Widal tube agglutination test was performed using a commercial kit (Murex Biotech Limited, UK) in accordance with the instructions of the manufacturers. Briefly, serum dilutions were made for each antigen to be tested using saline. One drop of bacterial suspension was added to each tube, the contents were mixed and incubated at 50°C for four and two hours for O and H suspensions respectively and agglutination was observed at the end of incubation.

Results

Table I shows comparison of results for detection of antibodies against H and O *Salmonella* antigens using the Widal tube test and the modified microplate agglutination assays for the 26 patients suspected to be suffering from *Salmonella* infection. Widal tube agglutination test detected H and O *Salmonella* agglutinins in 17 and 22 patients respectively. Microplate assay using 0.5% BSA as a blocking agent enhanced the level of detection of H and O *Salmonella* agglutinins but sta-

Table I
Comparison of Widal tube agglutination test with modified microplate agglutination assay in patients suspected to have typhoid fever

S. No.	Widal Tube test		Microplate Agglutination with 0.5% BSA		Microplate Agglutination SRBC absorption		Microplate Agglutination Heat inactivation	
	H titer	O titer	H titer	O titer	H titer	O titer	H titer	O titer
1	40	80	80	160	320	320	–	–
2	320	40	40	40	5120	80	–	–
3	–	80	80	80	40	160	–	–
4	–	80	80	80	80	160	–	–
5	–	80	80	80	80	160	–	–
6	320	40	40	80	1280	160	–	–
7	–	80	80	80	40	80	160	–
8	40	160	160	160	320	160	–	–
9	–	160	160	160	80	640	–	80
10	40	80	80	80	160	320	–	–
11	–	80	80	80	–	320	–	–
12	80	80	80	320	320	640	–	80
13	80	80	80	160	320	320	–	–
14	160	320	320	320	640	320	–	160
15	320	320	320	320	1280	640	160	320
16	40	80	80	160	80	320	320	40
17	160	–	–	–	640	80	40	–
18	640	–	–	–	5120	–	–	–
19	–	80	80	160	80	160	–	–
20	80	40	40	40	320	40	–	–
21	640	–	–	40	2560	40	–	–
22	160	–	–	–	640	–	–	–
23	–	80	80	80	80	80	–	80
24	–	40	40	80	–	160	80	–
25	320	80	80	80	2560	160	–	80
26	320	–	–	40	1280	40	80	–
p value			0.02	ns	0.005	0.001	0.03	0.01

– represents a negative test; ns = not significant
Diagnostic titers for H and O agglutinins were ≥ 1:160 and 1:80 respectively.

tistically significant difference was observed only for H agglutinins ($p \leq 0.02$) when compared with Widal tube agglutionation test. Absorption of sera with SRBC was also associated with significant enhancement of the detectable titers for both H ($p \leq 0.001$) and O ($p \leq 0.005$) *Salmonella* antigens when compared with the titers obtained by Widal tube agglutination test. Heat inactivation on the other hand had an opposite effect where both H ($p \leq 0.03$) and O ($p \leq 0.01$) agglutinin detection significantly decreased when compared with the Widal tube agglutination test. Table II shows the comparison of data from 11 patients with bacteriologically confirmed *Salmonella* infection. All the patients were found to have high titers of antibodies against O and H *Salmonella* antigens when tested with the Widal tube agglutination test. Comparative analysis revealed further enhancement in the titers for both H ($p \leq 0.0002$) and O ($p \leq 0.015$) agglutinins when sera samples were subjected to SRBC absorption. In the presence of 0.5% BSA significant enhancement was noted only for H agglutinins. Table III compares data for the percentages of positive tests for *Salmonella* agglutinins in 26 patients suspected to have *Salmonella* infection. Compared to the Widal tube agglutination test, the modified microplate agglutination test detected a higher percentage of positive tests for H and O *Salmonella* agglutinins, either in the presence of 0.5% BSA or when the sera were absorbed with SRBCs. Heat inactivation however was associated with decreased percentage of positive tests for both H and O *Salmonella* agglutinins.

Table II
Comparison of Widal tube agglutination test with modified microplate agglutination assay in patients with bacteriologically confirmed typhoid fever

S. No.	Widal Tube test		Microplate Agglutination with 0.5% BSA		Microplate Agglutination SRBC absorption		Microplate Agglutination Heat inactivation	
	H titer	O titer	H titer	O titer	H titer	O titer	H titer	O titer
1	2560	160	5120	160	10240	640	1280	160
2	320	160	640	320	5120	640	160	320
3	640	1280	1280	1280	5120	2560	1280	640
4	640	1280	5120	1280	10240	5120	1280	640
5	320	320	1280	320	2560	640	320	160
6	2560	320	5120	640	10240	1280	1280	320
7	2560	5120	10240	5120	20480	10240	1280	2560
8	2560	320	5120	640	20480	10240	640	640
9	640	640	2560	1280	10240	5120	320	640
10	640	640	1280	640	5120	1280	640	640
11	1280	160	2560	320	2560	2560	640	320
p value			0.009	ns	0.0002	0.01	ns	ns

ns = not significant
Diagnostic titers for H and O agglutinins were ≥ 1:160 and 1:80 respectively

Table III
Comparison of diagnostic titers for *Salmonella* agglutinins between Widal tube agglutination test and modified microplate agglutination assay in 26 patients with suspected typhoid fever

Type of test	*Salmonella* O agglutinins No. (%)	*Salmonella* H agglutinins No. (%)
Widal tube agglutination test	17 (65.3)	10 (38.4)
Microplate agglutination test using 0.5% BSA	19 (73)	15 (57.6)
Microplate agglutination test with SRBC absorption	21 (80.7)	16 (61.5)
Microplate assay with heat inactivation	7 (26.9)	8 (30.7)

Diagnostic titers for O and H agglutinins were ≥ 1:80 and 1:160 respectively.

Discussion

Using the novel microplate agglutination assay this study shows significant enhancement in the detection of titers for H and O *Salmonella* agglutinins both in the presence of 0.5% BSA and SRBC absorption, when compared with the results of the routine Widal tube agglutination test.

The presence of 0.5% BSA was shown to enhance the detection of *Salmonella* agglutinins. Since BSA as a blocking agent was used for the first time in the microplate agglutination assay, various concentrations of BSA diluted in normal saline were tested; BSA concentration of 0.5% was found to be optimal where a clear-cut well-defined and smooth settlement was obtained consistently. Since BSA is known for its ability to block vacant binding sites in microplate wells (Steinitz, 2000), this blocking action may have allowed all antigen and antibody molecules to react uniformly and could have contributed to enhancement in detection of titers for *Salmonella* agglutinins. A 0.5% BSA concentration has been previously shown to enhance the titers for *Salmonella* O agglutinins in the passive haemagglutination assay (Coovadia *et al.*, 1986), supporting the role for BSA as an enhancing agent in the detection of *Salmonella* agglutinins.

The role of BSA as an enhancing agent has already been documented under various settings. For example, Polymerase Chain Reaction (PCR) for DNA amplification is limited, in part, by the presence of inhibitors in biological samples that reduce the amplification efficiency. The addition of BSA to reaction mixtures of PCR has clearly been shown to decrease the inhibitory effect of blood and allow DNA amplification (Abu Al-Soud and Rådström, 2000). For the detection of *Mycobacterium tuberculosis* in respiratory specimens the presence of BSA in the PCR reaction has been regarded as mandatory (Forbes and Hicks, 1996). Similarly the presence

of BSA in ELISA also eliminates interference resulting from the use of human, rat or mouse serum albumins in the assay (Pestka and Chu, 1984). These data along with the findings of this study highlight the role of BSA as an amplification facilitator, though the exact mechanism of action remains obscure.

Significantly high titers of *Salmonella* agglutinins both for H and O antigens were detected using the microplate agglutination assay compared with those detected by the Widal tube agglutination test when serum samples were absorbed with SRBCs. Using non-motile *Salmonella typhi* organisms in a haemagglutination assay absorption of serum with SRBC has previously been shown to enhance the level of detection of O agglutinins, with no effect on detecting H agglutinins (Jesudason *et al.*, 1991). On the basis of this finding antibody response against the non-flagellar proteins was proposed to be more important in *Salmonella* infection. In disagreement with this proposition it is possible that the use of non-motile *Salmonella typhi* organisms may have resulted in subsequent precipitation of non-flagellar proteins only. The enhancement of both H and O agglutinin titers observed in the present study could be due to the fact that commercially prepared suspensions of both O and H antigens were used. The findings of both studies, however, are consistent with regards to enhancement in the level of detection of *Salmonella* agglutinins when the sera were absorbed with SRBCs prior to being tested.

SRBCs in routine laboratory practice are used to detect heterophile antibodies. These antibodies are characteristically found in the sera of patients suffering from infectious mononucleosis, along with other clinical conditions, where the serum levels of heterophile antibodies have also been related to disease activity (Duverlie *et al.*, 1989; Yoshida *et al.*, 1980; Satoh *et al.*, 1980; Moore and Dorner, 1980; Tamura *et al.*, 1984). Since heterophile antibodies can be detected in an otherwise healthy population (Kakoma *et al.*, 1987) their presence in the serum samples, sent to the laboratory for routine investigations and their ability to interfere with the results, cannot be ignored. There are no reliable data regarding heterophile antibodies in *Salmonella* infection. The findings of this study, however, provide indirect evidence that these antibodies could be present in the samples tested. The enhancement of titers and the percentage for *Salmonella* agglutinins, observed consequent to SRBC absorption, may be due to the depletion of heterophile antibodies in serum samples.

Heat inactivation was found to be associated with a decreased level of detection titers for *Salmonella* agglutinins. Using the indirect haemagglutination test, indirect fluorescent antibody test and enzyme-linked immunosorbent assay, heat inactivated serum samples yielding low titers of *Salmonella* agglutinins in patients with a confirmed diagnosis of typhoid fever has been reported in the past (Rai *et al.*, 1989); this was attributed to partial antibiotic therapy prior to presentation. Since reduction in the titers of *Salmonella* agglutinins, both for H and O antigens was noticed uniformly in this study, heat induced protein denaturation appears to be the most likely cause. This is evident from the fact that heating mouse serum has been shown to cause a selective loss of immunoglobulin isotypes (Schetters *et al.*, 1988). Alternatively this observation may indicate the presence of a thermolabile factor in the serum modulating antigen antibody interactions.

Conclusion. Findings of this study reveal that the novel modified microplate agglutination assay using the SRBC absorption technique, apart from being a useful alternative, performs better than the Widal tube agglutination test for the serological diagnosis of typhoid fever. It offers the advantages of simplicity, rapidity, economy and flexibility for handling a large numbers of specimens. This microplate assay appears to a useful assay not only for the detection of *Salmonella* infection but may be applied for diagnosis of other bacterial infections. This study was limited by relatively small number of patients investigations involving a larger group of patients are recommended to further evaluate the microplate assay.

Literature

Abu Al-Soud W. and P. Rådström. 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, Feces, and Meat. *J. Clin. Microbiol.* 38: 4463–4470.

Bakr W.M., L.A. El Attar, M.S. Ashour and A.M. El Toukhy. 2011. The dilemma of widal test – which brand to use? a study of four different widal brands: a cross sectional comparative study. *Ann. Clin. Microbiol. Antimicrob.* 10–17.

Baker S., M. Favorov and G. Dougan. 2010. Searching for the elusive typhoid diagnostic. *BMC Infect. Dis.* 10: 45–52

Barrett TJ., P.A. Blake, S.L. Brown, K. Hoffman, J. Mateullort and J.C. Feeley. 1983. Enzyme linked immunosorbent assay for detection of human antibodies to *Salmonella* Vi antigen. *J. Clin. Microbiol.* 17: 625–627.

Barsoum IS. and A.Y. Awad. 1972. Microtitre plate agglutination test for *Salmonella* antibodies. *Appl. Microbiol.* 23: 42–50.

Chart H., B. Rowe and S.S. Cheesbrough. 1997. Serological response of patients infected with *Salmonella typhi*. *J. Clin. Pathol.* 50: 944–946.

Chart H., D.J. Waghorn and B. Rowe. 1994a. Serological detection of patients infected with *Salmonella enteritidis. Serodiagn Immunother Infect. Dis.* 6: 79–81.

Chart H., D.J. Waghorn and B. Rowe. 1994b. Serological response of patients infected with *Salmonella enteritidis* PT4. *Serodiagn Immunother Infect. Dis.* 6: 21–24.

Choo K.E., A.R. Raazib, S.J. Oppenheimer, W.A. Ariffin, J. Lau and T. Abraham. 1993. Usefulness of the Widal test in diagnosing childhood typhoid fever in endemic areas. *J. Paedia. Child. Health.* 29: 36–39

Clegg A., M. Passey, M. Omena, K. Karigifa and N. Suve. 1994. Re-evaluation of the Widal agglutination test in response to the

changing pattern of typhoid fever in the highlands of papua New Guinea. *ACTA Tropica.* 57: 255–263.

Coovadia YM., V. Sing, R.H. Bhana and N. Moodley. 1986. Comparison of passive haemagglutination test with Widal agglutination test for serological diagnosis of typhoid fever in an endemic area. *J. Clin. Pathol.* 39: 680–683.

Duthie R. and G.L. French. 1990. Comparison of methods for the diagnosis of typhoid fever. *J. Clin. Pathol.* 43: 863–865.

Duverlie G., M. Driencourt, C. Roussel and J. Orfila. 1989. Heterophile IgM, IgA, and IgE antibodies in infectious mononucleosis. *J. Med. Virol.* 28: 38–41.

Farooqui B.J., M. Khurshid, M. K. Ashfaq and M. A. Khan. 1991. Comparative yield of *Salmonella typhi* from blood and bone marrow cultures in patients with fever of unknown origin. *J. Clin. Pathol.* 44: 258–9.

Forbes B.A. and K.E. Hicks. 1996. Substances Interfering with Direct Detection of *Mycobacterium tuberculosis* in Clinical Specimens by PCR: Effects of Bovine Serum Albumin. *J. Clin. Microbiol.* 34: 2125–2128.

Ghosh K., K.N. Javeri, D. Mohanty, B.D. Parmar, R.R. Surati and S.H. Joshi. 2001. False positive serological tests in acute malaria. *Br. J. Biomed. Sci.* 58: 20–3.

Hoffman SL., T.P. Flanigan, D. Klaucke, B. Leksana, R.C. Rockhill, N.H. Punjabi, S.P. Pulungsih, A. Sutomo and M.A. Moechtar. 1986. Widal slide agglutination test, a valuable rapid diagnostic test in typhoid fever patients at the infectious diseases Hospital of Jakarta. *Am. J. Epidemiol.* 123: 869–875.

Jesudason MV., G. Sridharan, R. Arulselvan, P.G. Babu, PG and T.J. John. 1998. Diagnosis of typhoid fever by the detection of anti-LPS and anti-flagellin antibodies by ELISA. *Indian. J. Med. Res*. 107: 204–7.

Jesudason MV., G. Sridharan, M. Santhossh and T.J. Lohn. 1991. Measuring antibody to non-flagellar proteins of *Salmonella typhi* for diagnosis of typhoid fever. *Trans. R. Soci. Trop. Med. Hyg.* 85: 117–118.

Jindal N., I.B. Aggarwal, Arora. A and S. Arora. 1992. Diagnostic accuracy of IgM antibody detection in typhoid by a modification in Widal test. *Indian. J. Med. Res*. 95: 224–226.

Kakoma I., R.M. Mwendapole, M. Bulsara, S. Mabenga, C.S. Syabula and K.F. Wurapa. 1987. Profiles of heterophile antibody to various mammalian erythrocytes in rural populations of Zambia. *Comp. Immunol. Microbiol. Infect. Dis.* 10: 51–57.

Keddy KH., A. Sooka, M.E. Letsoalo, G. Hoyland, C.L. Chaignat and A.B. Moore TL. and R.W. Dorner. 1980. 19S IgM Forssman-type heterophile antibodies in juvenile rheumatoid arthritis. *Arthritis. Rheum*. 23: 1262–1267.

Morrissey, J.A. Crump. 2011. Sensitivity and specificity of typhoid fever rapid antibody tests for laboratory diagnosis at two sub-Saharan African sites. *Bull. World. Health. Organ*. 89: 640–647.

Nardiello S., T. Phizzella, M. Russo and B. Galanti. 1984. Serodiagnosis of typhoid fever by enzyme linked immunosorbent assay of anti-*Salmonella typhi* lipopolysaccharide antibodies. *J. Clin. Microbiol.* 20: 718–721

Omuse G., R. Kohli and G. Revathi. 2010. Diagnostic utility of a single Widal test in the diagnosis of typhoid fever at Aga Khan University Hospital (AKUH), Nairobi, Kenya. *Trop. Doct*. 40: 43–44.

Pang T. and S.D. Putucheary. 1983. Significance and value of the Widal test in the diagnosis of typical fever in an endemic area. *J. Clin. Pathol.* 36: 471–475.

Parry C.M., T.T.H. Nguyen, D. To Song, W. John and T.C. Nguyen. 1999. Value of a single-tube Widal test in diagnosis of typhoid fever in Vietnam. *J. Clin. Microbiol*. 37: 2882–2886.

Pestka J.J. and F.S. Chu. 1984. Aflatoxin B1 dihydrodiol antibody: production and specificity. *Appl Environ Microbiol*. 47: 472–477.

Rai GP., K. Zachariah and S. Shrivastava. 1989. Comparative efficacy of indirect haemagglutination test, indirect fluorescent antibody test and enzyme linked immunosorbent assay in serodiagnosis of typhoid fever. *J. Trop. Med. Hyg*. 92: 431–434.

Saha S.K., M. Ruhulamin, M. Hanif, M. Islam and W.A. Khan. 1996. Interpretation of the Widal test in the diagnosis of typhoid fever in Bangladeshi children. *Ann. Trop. Peadiatr.* 16: 75–78.

Satoh PS., A.J. Elberg, W.E. Fleming, H.J. Baluarte and A.B. Gruskin. 1980. Heterophile antibodies in the serum of children with nephrotic syndrome. *Vox. Sang*. 39: 128–133.

Schetters T., J. Van Run-Van Breda, A. and W. Eling. 1988. Selective depletion of immunoglobulin isotypes after decomplementation of mouse sera by heat treatment. *J. Immunol. Methods.* 109: 193–197.

Sharma M., U. Datta, P. Roy, S. Verma and S. Sehgal. 1997. Low sensitivity of counter-current immuno-electophresis for serodiagnosis of typhoid fever. *J. Med. Microbiol*. 46: 1039–1042.

Steinitz M. 2000. Quantitation of the blocking effect of tween 20 and bovine serum albumin in ELISA microwells. *Anal. Biochem.* 282: 232–238.

Tamura T., F. Merino, K. Kano, P. Ramirez-Duque and F. Milgrom. 1984. Heterophile antibodies in sera of patients with Chediak-Higashi syndrome. *Int Arch Allergy Appl Immunol*. 73: 186–188.

Verdugo-Rodriguez A., Y. Lopez-Vidal, J.L. Puente, G.M. Ruiz-Placios and E. Calva. 1993. Early diagnosis of typhoid fever by Enzyme Immunoassay using *Salmonella typhi* outer membrane protein preparation. *Eur. J. Clin. Microbiol. Infect. Dis*. 12: 248–254.

Wain J. and S. Hosoglu. 2008. The laboratory diagnosis of enteric fever. *J. Infect. Dev. Ctries*. 2: 421–5.

Yoshida H., N. Takeda, T. Nishimaki, T. Morito, R. Kasukawa, K. Kano and F. Milgrom. 1980. Heterophile antibodies in Japanese patients with various diseases including infectious mononucleosis. *Immunol. Commun.* 9: 403–408.