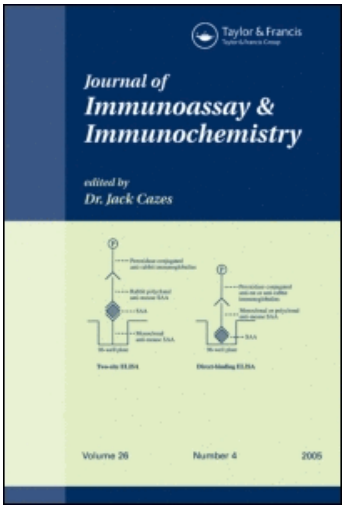

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Sandwich Enzyme Immunoassays for Detection of Salmonella Typhi

Ayub Qadri[abcd]; Souravi Ghosh[abcd]; Kunti Prakash[abcd]; Ramesh Kumar[abcd]; Kamal D. Moudgil[abcd]; Gursaran P. Talwar[abcd]

[a] National Institute of Immunology, New Delhi [b] Department of Microbiology, Lady Hardinge medical College, New Delhi [c] Department of Microbiology, All India Institute of Medical Sciences, New Delhi [d] Department of Biochemistry, All India Institute of Medical Sciences, New Delhi

To cite this Article Qadri, Ayub , Ghosh, Souravi , Prakash, Kunti , Kumar, Ramesh , Moudgil, Kamal D. and Talwar, Gursaran P.(1990) 'Sandwich Enzyme Immunoassays for Detection of Salmonella Typhi', Journal of Immunoassay and Immunochemistry, 11: 2, 251 — 269

To link to this Article: DOI: 10.1080/01971529008053272

URL: http://dx.doi.org/10.1080/01971529008053272

JOURNAL OF IMMUNOASSAY, 11(2), 251-270 (1990)

SANDWICH ENZYME IMMUNOASSAYS FOR DETECTION OF *Salmonella typhi*.

Ayub Qadri, Souravi Ghosh, Kunti Prakash, Ramesh Kumar, Kamal D. Moudgil and Gursaran P. Talwar.
National Institute of Immunology,New Delhi; Department of Microbiology, Lady Hardinge medical College, New Delhi; Department of Microbiology, All India Institute of Medical Sciences, New Delhi; Department of Biochemistry, All India Institute of Medical Sciences, New Delhi.

ABSTRACT

Enzyme immunoassays were developed using monoclonal antibodies raised against somatic (O), flagellar (H) and capsular (Vi) antigens of *Salmonella typhi*. The assay based on anti-O monoclonal antibodies could specifically detect *S.typhi* and soluble lipopolysaccharide (LPS) isolated from *S.typhi*. Anti-H MoAbs detected motile *S.typhi* and soluble flagellar antigen. Monoclonal antibodies against capsular polysaccharide could detect Vi-containing *S.typhi* as well as soluble Vi antigen. The three assays reported here detected *S.typhi* with 100% sensitivity in blood culture broths obtained from bacteriologically confirmed typhoid patients and were negative with blood specimens containing *Salmonella senftenberg*, *E.coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* or *Streptococcus* (α-hemolytic) derived from patients with pyrexia. The assays, however, did not demonstrate the presence of soluble antigens in sera and urine samples obtained from typhoid patients. (KEY WORDS : Monoclonal antibody - MoAb, enzyme immunoassay - EIA, *Salmonella typhi*).

Address for correspondence:
Ayub Qadri
National Institute of Immunology,
New Delhi - 110067.

INTRODUCTION

Typhoid fever continues to be one of the major infectious diseases in many countries of the world and poses a serious public health problem (1,2). A rapid laboratory diagnosis is essential for prompt treatment of the disease. Currently the most definitive method of diagnosis of typhoid is the detection of S.typhi in the blood or in other biological fluids by culture methods. However, confirmatory identification of the organism takes two or more days by conventional bacteriological techniques. Widal's agglutination test, a commonly used diagnostic test for serum antibodies is positive only one week after the onset of the disease and lacks specificity. Because of high endemicity of the disease, agglutinins are frequently found in the sera of normal healthy people and febrile non-typhoidal subjects (3). Thus there is need for a test which would detect specifically S.typhi antigens (and not the antibodies). A number of studies have shown the presence of S.typhi antigen(s) in the sera of patients suffering from typhoid fever (4,5,6). The presence of Vi capsular polysaccharide in the urine of typhoid patients has been demonstrated by Rockhill et al. (7) and Barrett et al. (8). However, attempts to use such an antigen based test for definite diagnosis of typhoid fever have not been very successful mainly due to lack of specific antibodies (9). These assays employed polyclonal antibodies which often have low specificity due to extensive cross reactivity amongst the gram negative bacteria. We have recently reported generation of a number of monoclonal antibodies against somatic, flagellar and capsular

antigens of S.typhi (10,11,12). In this communication we describe enzyme immunoassays employing these monoclonal antibodies, which enable the detection of S.typhi with high sensitivity and specificity in blood cultures of typhoid patients.

MATERIALS AND METHODS

Bacteria

The flagellar strain of S.typhi-901 was obtained from Central Research Institute, Kasauli, India. Vi positive strain of S.typhi was an isolate from a typhoid patient obtained from Lady Hardinge Medical College, New Delhi. Aflagellar S.typhi-901 was obtained from the Department of Microbiology, All India Institute of Medical Sciences, New Delhi. The microorganisms were grown in bacto tryptone-yeast extract (Difco Laboratories, Det.,USA) - saline broth overnight at 37^0C, harvested, washed with saline and suspended in saline. Bacteria were counted by nephelometry.

Clinical specimens

Blood culture broths of patients were obtained from the Microbiology Department of Lady Hardinge Medical College (LHMC), New Delhi. Sera and urine samples were provided by the Microbiology Department, All India Institute of Medical Sciences (AIIMS), New Delhi.

Antigens

Lipopolysaccharide (LPS) and flagellar antigen (H) were isolated and purified from S.typhi by the methods of Westphal & Jann (13) and Ibrahim et al. (14) respectively. Purified Vi capsular polysaccharide isolated from Citrobacter freundii

was provided by Dr. John B.Robbins, National Institutes of Health, Bethesda.

Antibodies

The production and characteristics of monoclonal antibodies against LPS, H-antigen and Vi have been reported elsewhere (10,11,12).

Polyclonal antibodies against H antigen were raised by immunizing New Zealand White rabbits subcutaneously with $30x10^6$ of formalin fixed S.typhi-H suspension (obtained from Central Research Institute, Kasauli, India) emulsified with incomplete Freund's adjuvant (IFA). Rabbits were boosted intramuscularly with $15x10^6$ of the same suspension in IFA at 3-4 week intervals. Bleeds when analysed for antibody against purified flagellar antigen by a solid phase enzyme immunoassay, had a titre of 10^{-5} (data not shown).

Purification of antibodies

Polyclonal (R114) and monoclonal (P3A2B11/B12) antibodies against flagellar antigen and monoclonal anti-O antibodies P3B2F7 and P1C4C9 were purified by affinity chromatography on Protein A-Sepharose (obtained from Pharmacia chemical Co., Sweden) column by the method of Ey et al.(15).

Anti-O monoclonal antibody P5C6D1 (IgG1) and anti-Vi MoAbs P2C2D5/H5 (IgM) and P2B1G2/A9 (IgG1) were partially purified from ascitic fluids by precipitation with cold ammonium sulphate, suspended in PBS (50 mM phosphate, 150mM sodium chloride, pH 7.4) and dialysed extensively against PBS. These were used without any further purification.

Conjugation of antibodies to enzyme horse radish peroxidase (HRP):

MoAbs P1C4C9, P5C6D1, P3A2B11/B12 and P2B1G2/A9 were coupled to HRP by periodate oxidation method described by Nakane and Kawaoi (16). P3B2F7 was conjugated by two step glutaraldehyde method described by Avrameas (17).

Sandwich Enzyme linked immunosorbent assays

Different combinations of monoclonal antibodies were employed as capture and revealing antibodies as described in Figs 1&2, 3 and 4 for O, H and Vi antigens respectively. The capture antibodies diluted in carbonate buffer (50 mM, pH 9.5) were coated at optimum protein concentrations (standardised by checker board ELISAs; 20µg/ml for P2C2D5/H5, 25 µg/ml for P1C4C9, P5C6D1 & P3A2B11/B12, 50µg/ml for R114 and 100 µg/ml for P3B2F7) onto the polyvinyl microtitre plates overnight at 37^0C. After washing with PBS-Tween (PBS containing 0.05% Tween-20), non-specific sites were blocked with 2% bovine serum albumin (Cohn's fraction v from Sigma chemical Co.) in PBS for 1hr at 37^0C. Plates were washed and incubated for 1 hr with different concentrations of soluble antigens or bacteria. Control wells had no antigen or bacteria. After washing with PBS-Tween, plates were incubated with HRP-labelled monoclonal antibodies diluted in PBS containing 1% BSA and 0.05% Tween, for 1 hr at 37^0C. Plates were thoroughly washed with PBS-Tween and the enzyme activity was determined by adding 100 µl/well of freshly prepared substrate solution

(0.5 mg ortho phenylene diamine dissolved in 50mM citrate phosphate buffer - pH 5.4). The reaction was stopped after 5 min by adding 50 µl/well of 5N H_2SO_4 and the absorbance measured at 490 nm in a Biotek ELISA Reader.

The effect of serum, blood and urine on the sensitivity of the assays was studied by dispensing the soluble antigens in serum or urine (LPS and H antigen in the serum and Vi in the urine) and bacteria in bacterial culture medium containing normal human blood (10% final concentration).

The sensitivity of the assays was also studied by inoculating about 0.9 CFU of S.typhi/ml in 5 ml of bacterial culture medium consisting of bacto-tryptone, yeast extract and sodium chloride and screening the cultures at different time intervals by the three assays described for detection of S.typhi.

Detection of S.typhi and soluble antigens in clinical specimens:

Blood culture broths from patients diagnosed clinically as cases of enteric fever, septicemia and pyrexia of unknown origin were taken at incubation time periods ranging from 6 hrs to 24 hrs, for EIA. The cultures were independently processed by the Department of Microbiology and characterised for the microorganism. EIAs were conducted on coded samples prior to microbiology results. 20 sera and 25 urine samples obtained from typhoid patients were screened for antigens O, H and Vi.

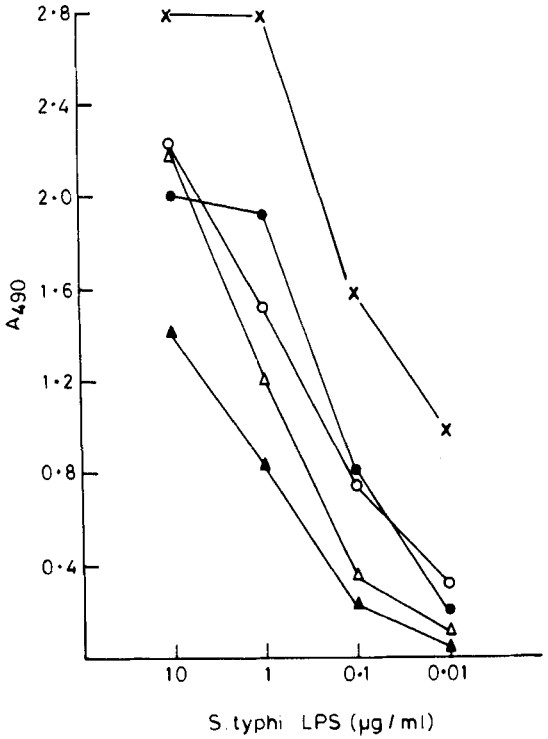

FIGURE 1: ELISA for detection of S.typhi LPS.

●——● Capture antibody P1C4C9, Revealing antbody P1C4C9

o——o Capture antibody P5C6D1 -

△——△ Capture antibody P3B2F7 -

x——x Capture antibody P1C4C9, Revealing antibody P5C6D1

▲——△ Capture antibody P1C4C9, Revealing antibody P3B2F7

RESULTS

Three monoclonal antibodies P1C4C9, P5C6D1 and P3B2F7 had high specificity for serogroup D, D & B and D, A & B Salmonellae respectively. These were devoid of reactivity with a variety of gram negative bacteria as described

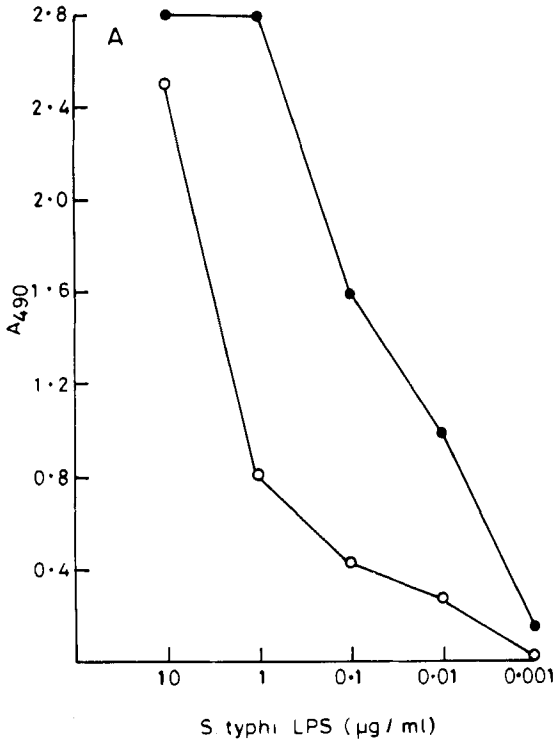

FIGURE 2: A-ELISA for detection of S.tyhpi LPS.
Capture antibody P1C4C9, Revealing antibody P5C6D1
●——● LPS diluted in 1% BSA, o——o LPS diluted in normal human serum.
B-ELISA for detection of S.typhi.
●——● Bacteria diluted in medium, o——o Bacteria in medium containing normal human blood.

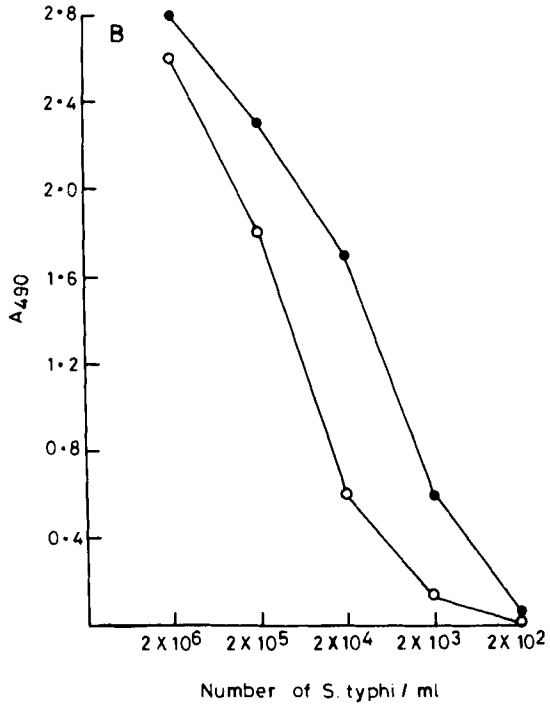

FIGURE 2, continued.

elsewhere (10). EIAs were designed using different combinations of these three MoAbs as capture and revealing antibodies. The antigens recognized by these MoAbs were O-antigenic determinants 9 and 12. The initial standardisation was thus carried out with LPS isolated from S.typhi. Fig 1 gives the results. Although all combinations were usable, a higher sensitivity was obtained with P1C4C9 as capture and P5C6D1 as the revealing antibody and this pair of MoAbs was selected for further investigations. It was important to

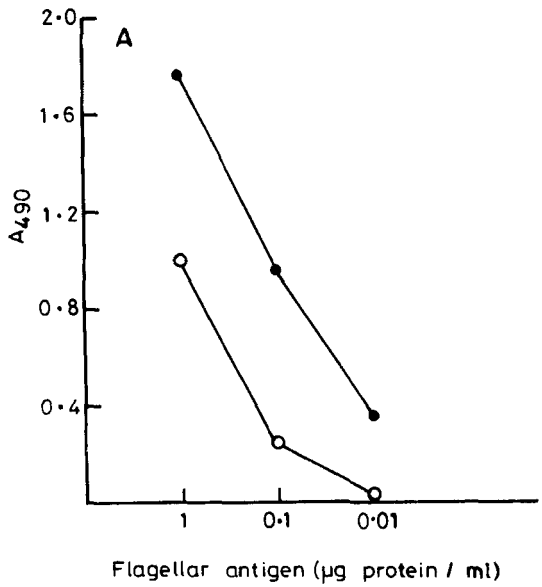

FIGURE 3: A-ELISA for detection of soluble flagellar antigen.

●——● Capture antibody R114, Revealing Ab P3A2B11/B12.

o——o Capture antibody P3A2B11/B12, -

B-ELISA for detection of motile S.typhi

o——o Capture antibody R114, Revealing Ab P3A2B11/B12

●——● Capture antibody P3A2B11/B12, -

determine the effect of blood components on the detection of S.typhi LPS and whole bacteria in the EIA. Another set of assays was thus performed by dispensing LPS in normal human serum and bacteria in culture medium containing normal human blood. A quenching effect was observed in both the cases. The assay could detect upto 10ng/ml of LPS in serum and

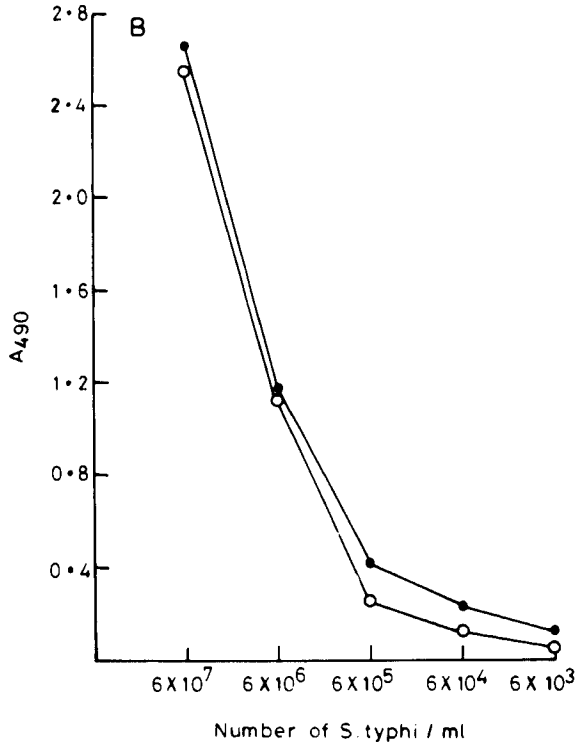

FIGURE 3, continued

$2x10^3$bacteria/ml dispensed in medium containing normal human blood (Fig2 A,B)

There have been reports on the presence of soluble antigens H, Vi and O in sera and urine of patients besides the bacilli. EIAs were thus also developed for H and Vi antigens. For H, the MoAb P3A2B11/B12 could be employed on both sides of the sandwich. The sensitivity of this combination for free

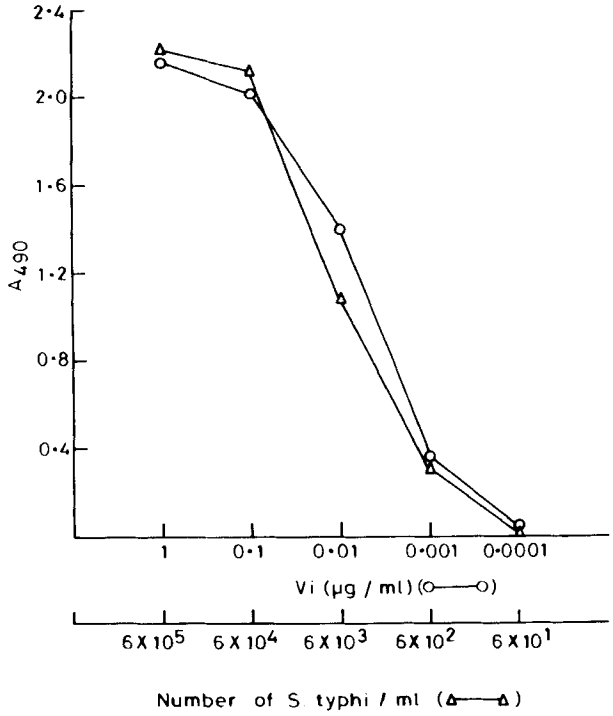

FIGURE 4: ELISA for detection of Vi and Vi containing S.typhi
Capture antibody P2C2D5/H5, Revealing antibody P2B1G2/A9
o——o Vi diluted in normal human urine,
Δ——Δ S.typhi in medium containing blood.

H antigen was a little lower than an assay where a polyvalent high titre rabbit antibody was employed as capture and P3A2B11/B12 as the revealing (Fig 3-A). However, the MoAb combination was as good as the polyclonal-monoclonal combination for detection of motile S.typhi (Fig 3-B). The

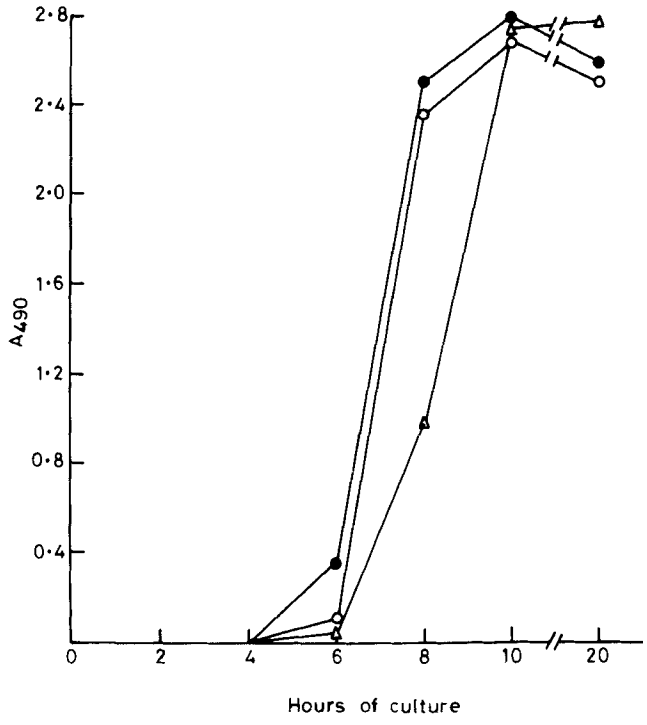

FIGURE 5: ELISA for detection of S.typhi in live cultures at different itme intervals.

●——● O-ELISA, △——△ H-ELISA, ○——○ Vi-ELISA

assays could detect upto 10 ng/ml of H antigen and $6x10^3$ flagellar S.typhi/ml.

Assay for Vi was developed employing an IgM MoAb P2C2D5/H5 as capture and an IgG MoAb P2B1G2/A9 as revealing. The assay estimated Vi dispensed in normal human urine as well as S.typhi dispensed in culture medium containing normal human

TABLE 1

Detection of S.typhi in clinical specimens.

	No.positive/ No.tested (% positive)		
	O-ELISA	H-ELISA	Vi-ELISA
S.typhi	102/102 (100)	48/48(100)	63/63 (100)
S.senftenberg	0/20 (0)	N.D	N.D
E.coli	0/51 (0)	N.D	0/11 (0)
K.pneumoniae	0/33 (0)	0/33 (0)	0/33 (0)
P.aeruginosa	0/10 (0)	0/5 (0)	0/5 (0)
Proteus mirabilis	0/6 (0)	N.D	N.D
Streptococcus (α-hemolytic)	0/11 (0)	N.D	N.D

N.D - not done.

blood. The sensitivity was <1ng/ml Vi and $6x10^2$bacteria/ml respectively (Fig 4).

The sensitivity of the assays was also determined by screening S.typhi cultures at different time intervals after inoculation (Fig 5). The O and Vi-EIAs could detect bacteria after 5-6 hrs of inoculation.

Analysis of clinical samples

The studies were carried out on coded samples of blood culture broths supplied by the Department of Microbiology, LHMC. 233 culture broths were analysed by O-ELISA, 86 by H-ELISA and 112 by Vi-ELISA. The results are given in Table 1.

Each EIA gave comparable results. The results of the EIAs were confirmed subsequently by bacteriological culture. There was 100% correlation between the EIAs and bacterial culture. The assays were also highly specific and none of the bacteria other than S.typhi detected by culture in blood specimens, were picked up by the EIAs. However,none of the sera or urine samples from typhoid patients showed the presence of soluble O, H and Vi antigens when analysed by these assays.

DISCUSSION

The use of antibodies for rapid identification of Salmonella species in culture and in clinical specimens has been reported earlier (18-23). With the exception of one (23), all other assays used polyclonal antibodies which often have cross reaction with other enterobacteria and hence lack specificity. Lim and Fok (23) used an anti-O9 MoAb based agglutination assay for detection of group D Salmonellae. As the authors have themselves pointed out, a limitation of the O 9 system is that infections caused by S.typhi can not be distinguished from those caused by other commonly occuring group D Salmonellae such as S.enteritidis and the only way to achieve that is by showing the presence of d-H and Vi antigens in S.typhi organisms. In the present study, EIAs were developed for not only O antigens but also H and Vi employing MoAbs of high specificity. Although the EIAs for either O, H and Vi could detect S.typhi in clinical specimens, the use of two of these three assays would provide conclusive diagnosis and avoid possible wrong conclusion due

to cross reaction of antibodies with S.enteritidis, S.dublin or other group D Salmonellae which share similar O antigenic determinants.

The present EIAs have a sensitivity of detecting 10^3-10^4 S.typhi/ml. This concentration of bacilli is obtained after 6 hrs of culture of a sample containing 1 bacterium/ml (Fig 5). The inability of Vi-ELISA to detect S.typhi at an earlier time point inspite of its better sensitivity was probably due to low level expression of Vi in the initial phase of bacterial growth. Variations in the expression of Vi under varying conditions of growth have been reported as long back as 1934 by Felix and Pitt (24). Vi antigen did not mask the reactivity of anti-O monoclonal antibodies, probably due to the presence of an incomplete capsule or to differential expression of Vi on the bacteria.

The three assays (O-ELISA, H-ELISA & Vi-ELISA) detected S.typhi in 100% of the blood culture broths obtained from typhoid patients. The results were confirmed by bacterial culture. The tests did not pick up any sample containing S.enteritidis, E.coli, K.pneumoniae, Proteus mirabilis, P.aeruginosa or Streptococcus (α-hemolytic) thus exhibiting 100% specificity. However, none of these assays demonstrated the presence of soluble antigens in sera and urine samples from typhoid patients. The presence of circulating and excretory antigens in typhoid patients has been reported earlier by many workers (4,5,6). However, results have been conflicting. For example Taylor et al.(9) reported that an enzyme immunoassay and an agglutination assay for Vi antigen

employing polyclonal antibodies detected only 62.4% & 34% respectively of typhoid patients and also picked up cases with paratyphoid fever (64.7% & 48%), non-typhoidal febrile illnesses (47.6% & 46%) and normal healthy controls (13.2% & 14%). Better results had been reported by Rockhill et al. (7). Failure of our assays to detect soluble antigens in sera or urine samples from typhoid patients could be attributed either to low level of antigenemia or antigenuria in these patients (less than the detection limit of our assays) or as suggested by Taylor (9) for Vi, to antigens getting physically or chemically changed before these appear in the serum or urine.

The present assays have been further simplified and the assay time reduced from 2 hrs to 15-30 minutes by coincubating the antigen and the second antibody (data not shown). These assays enable the detection of S.typhi in clinical specimens after about 6-7 hrs of enrichment with the culture medium. By virtue of the properties of MoAbs employed, these assays have high sensitivity and specificity and ensure definite diagnosis of typhoid fever on the very day of taking of the blood sample. The assays could also be useful for screening foods for group D, A and B Salmonellae including S.typhimurium (P5C6D1 and P3B2F7 react with S.typhimurium also - unpublished data) and biological fluids from patients suffering from food poisoning due to these bacteria.

ACKNOWLEDGMENT

This work was supported by a grant from UNDP (IND/85/083/A/01/14). We thank Prof S.C.Chutani, University

College of Medical Sciences, New Delhi, Dr.P.Pillai (LHMC) and Mr Nitesh Mohan (AIIMS) for providing clinical samples; Mrs S.Sharma and Mr M.Singh, LHMC, for doing blood cultures.

REFERENCES

1. Edelman, R and Levine, M. M. Summary of an international workshop on typhoid fever. Rev. Infect. Dis. 1986; 8: 329-349.

2. Hornick, R. B. Selective primary health care, strategies for control of diseases in developing world XX Typhoid fever. Rev. Infect. Dis. 1985; 7: 536.

3. Levine, M. M., Grados, O., Gilman, R. H., Woodward, W. E., Solisplaza, R. and Waldman, W. Diagnostic value of widal test in areas endemic for typhoid fever. Am. J. Trop. Med. Hyg. 1978; 27: 795-800.

4. Gupta, A. K. and Rao, K. M. Simultaneous detection of Salmonella typhi antigen and antibody in serum by counter immunoelectrophoresis for an early and rapid diagnosis of typhoid. J. Immunol. Methods 1979; 30: 349-353.

5. John, T. J., Sivadasan, K. and Kurien, B. Evaluation of passive bacterial agglutination for the diagnosis of typhoid fever. J. Clin. Microbiol. 1984; 20: 751-753.

6. Sundarraj, T., Ilango, B. and Subramaniam, S. A study on the usefulness of counter immunoelectrophoresis for the detection of Salmonella typhi antigen in the sera of suspected cases of enteric fever. Trans. R. Soc. Trop. Med. Hyg. 1983; 77: 143-147.

7. Rockhill, R. C., Rumans, L. W., Lesmana, M. and Dennis, D. T. Detection of Salmonella typhi D, Vi and d antigens, by slide coagglutination , in urine from patients with typhoid fever. J. Clin. Microbiol. 1980; 11: 213-216.

8. Barrett, T. J., Snyder, J. D., Blake, P. A. and Feeley, J. C. Enzyme linked immunosorbent assay for detection of Salmonella typhi Vi antigen in urine from typhoid patients. J. Clin. Microbiol. 1980; 15: 235-237.

9. Taylor, D. N., Harris, J. R., Barrett, T. J., Hargrett, N., Praentzell, I., Valdivieso, C., Palomino, C., Levine, M. M.and Blake, P. A. Detection of urinary Vi antigen as a diagnostic test for typhoid fever. J. Clin. Microbiol. 1983; 18: 872-876.

10. Qadri, A., Gupta, S. K. and Talwar, G. P. Monoclonal antibodies delineate multiple epitopes on the O-antigens of Salmonella typhi lipopolysaccharide. J. Clin. Microbiol. 1988; 26: 2292-2296.

11. Qadri,A., Ghosh, S., Upadhay, S. and Talwar, G.P. Monoclonal antibodies against flagellar antigen of Salmonella typhi. Hybridoma 1989; 8: 353-359.

12. Qadri, A., Ghosh, S. and Talwar, G. P. Monoclonal antibodies against two discrete determinants on Vi capsular polysaccharide. Submitted for publication.

13. Westphal, O and Jann, K. Extraction with phenol-water and further applications of the procedure. Methods in Carbohydr. Chem. 1965; 5: 83-91.

14. Ibrahim, G.F., Fleet, G. H., Lyons, M. J. and Walker, R. A. Method for isolation of highly purified Salmonella flagellins J.Clin. Microbiol. 1985; 22:1040-1044.

15. Ey, P. L., Prowse, S. J.and Jenkins, C. R. Isolation of pure IgG1,IgG2a and IgG2b immunoglobulins from mouse serum using Protein A-Sepharose. Immunochemistry 1978; 15: 429.

16. Nakane,P.K. and Kawaoi, A. Peroxidase-labelled antibody. A new method of conjugation. J. Histochem.Cytochem. 1974; 22:1084.

17. Avrameas,S. Coupling of enzymes to proteins with glutaraldehyde. Use of conjugates for detection of antigens and antibodies. Immunochemistry 1969; 6: 43.

18. Araj, G. F. and Chugh, T. S. Detection of Salmonella species in clinical specimens by capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. 1987; 25: 2150-2153.

19. McRill, C. M., Kraner, T. T. and Griffith, R.W. Application of peroxidase-antiperoxidase assay to the identification of salmonellae from pure cultures and animal tissues. J. Clin. Microbiol. 1984; 13: 281-284.

20. Mikhail, I. A., Sanborn, W. R. and Sippel, R. W. Rapid, economical diagnosis of enteric fever by a blood clot culture coagglutination procedure. J. Clin. Microbiol. 1983; 17: 564-565.

21. Svengusson, B., Jorbeck, H. and Lindberg , A. A. Diagnosis of salmonella infections; specificity of indirect immunofluorescence for rapid identification of Salmonella enteritidis and usefulness of enzyme-linked immuosorbent assay. J. Infect. Dis. 1979; 140: 927-936.

22. Sanborn, W. R., Lesmana, L.and Edwards, E. A. Enrichment culture coagglutination test for rapid, low cost diagnosis of salmonellosis. J. Clin. Microbiol. 1980; 12: 151-155.

23. Lim, P-l and Fok, Y-P. Detection of Group D Salmonellae in blood culture broth and of soluble antigen by tube agglutination using an O-9 monoclonal antibody latex conjugate. J. Clin. Microbiol. 1987; 15: 1165-1168

24. Felix, A and Pitt, R. M. A new antigen of B.typhosus. Lancet 1934; ii: 186-191