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PROGRESS REPORT NO. 7

PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER

A RESEARCH PROJECT

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SUBMITTED BY

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PROJECT PROFILE

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BACKGROUND

Enteric fever (typhoid and paratyphoid fever) still remains a major but often underestimated public health problem in less developed countries (LDC). Part of the explaination of this underestimation is due to the difficulties of accurate diagnosis by the Widal test and non-availability of confirmatory hemoculture in much of the rural tropics. In improving the situation, a low cost, simple, rapid and accurate diagnostic test that can be afforded to be applied widely and can be carried out in peripheral health care facilities must be devised such that accurate diagnosis can be made and proper treatment given and that a more realistic epidemiological picture can be monitored.

This diagnostic test should represent an improvement over the standard Widal test and hemoculture, i.e., no cross reaction with other enterobacteriaceae which cause enteric fever like illnesses, the requirement of single specimen in stead of paired specimens, and accurate result in the patients with clinically proved enteric fever but have false negative hemoculture. Hence, this test should provide confident diagnosis with a single sample, preferrably in a rapid, inexpensive and simple way. A logical approach is that it should be accurate, sensitive and specific detection of the bacterium produ ts.

Several groups of investigators have tried several immunological techniques, i.e., coagglutination (1,2), ELISA (3,4) etc, for the detection of bacterial antigens in serum, urine and other body fluids. Unfortunately all of the

efforts in this direction have met with the problems of precision, reproducibility and cross reactivities with other enterobacteriaceae. This problems can be attributed mainly to the antibodies used as only conventional polyclonal antibodies have been employed. The variation of bacterial strains used to produce antisera and the uncertainty of absorption procedures in reducing cross-reactivities make it almost impossible to standardize such polyclonal antisera and the respective tests.

For accurate and specific detection of antigens of Salmonellae causing enteric fever, a secure and consistent source of antibodies will be required. Properly defined clones of hybridoma producing monoclonal antibodies to selected epitopes present a rational approach to meeting these requirements. Once such clones are characterized and established, production of reagents of consistent quality can be ensured and standard kits for a rapid, sensitive, specific and inexpensive diagnostic test, i.e., ELISA can be produced and the molecular components of the antigenic structure among these four species of Salmonellae which are specific to corresponding established monoclonal antibody can also be defined.

OVERALL OBJECTIVES

I. To produce mouse monoclonal antibodies (mAb) directed to species specific antigens of *S.typhi*, *S.paratyphi A*, *S.paratyphi B*, *S.paratyphi C* and common antigens among them but not cross reacting with other enterobacteriaceae that commonly cause enteric fever like illnesses.

II. To develop a rapid, sensitive, specific and inexpensive immunological test detecting salmonella antigens in urine and sera for the diagnosis of enteric fever and identification of the causative species for epidemiological monitoring.

III. To produce test kits that are simple and suitable for use even in rural areas of LDC where scientific equipments are not readily available.

IV. To identify molecular components of the antigenic structure of salmonellae which are specific to mAb produced in I.

OBJECTIVES OF THIS PERIOD

I. To develop and produce mouse monoclonal antibodies to *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C*, and to identify molecular components of antigens which are specific to the established mAbs (cont.).

II. To establish ELISA test kits (cont.).

OBJECTIVE I : To develop and produce mouse monoclonal antibodies to *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C*, and to identify molecular components of antigens which are specific to established mAbs (cont.).

MATERIALS AND METHODS

We have performed another 2 fusions. The antigen we have used for immunization of BALB/c J mice were purified protein antigen of *S.paratyphi A*, *S.paratyphi B*, *S.paratyphi C* (the preparation of these antigens were described in progress report No. 6).

The procedures of fusion, screening, identification, isotypic determination of mAbs, immunoblotting for mAbs specificity and identification of molecular components of specific antigens were the same as progress report No. 3.

RESULTS AND DISCUSSION

We have selected total of 95 hybrids. Seventy-two hybrids were specific to *S.paratyphi A*, nine hybrids were specific to *S.paratyphi B* and 14 hybrids were specific to *S. paratyphi C*.

S.paratyphi A The 72 hybrids were divided into 4 groups according to their isotypes, i.e. 57 hybrids were IgG1K., 9 hybrids were IgG2aK, 4 hybrids were IgG2bK and 2 hybrids were IgG3K. We picked up 1 hybrid from each group as a representative (Table 1). The immunoblotting for confirmation of specificity and identification of molecular components of their specific antigens are shown in Fig. 1.

Our results revealed that all 4 representative mAbs were very specific to *S.paratyphi A*, no cross reaction to another enterobacteriaceae.

We have compared 2 different preparations of *S. paratyphi A* antigen in the immunoblotting. One was the Barber protein (the method of preparation was described in progress report No.2) and the other was cruded antigen extracted from viable bacterial cells (grown on BHI agar) which was prepared by boiling the viable bacterial cells with sample buffer using in SDS-PAGE (containing 0. 0625 M Tris-HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol and 5% v/v 2-mercaptoethanol). The results are shown in Fig. 4 that the specific epitope of *S.paratyphi A* was degraded in the Barber protein preparation but was not in the cruded antigen extracted. Hence, the specific epitope of *S.paratyphi A* which our mAbs could recognize is the protein which has molecular weight of 52 Kd. We have found that, this 52 Kd band was one of the prominent bands in the immunoblotting when using homologous polyclonal antiserum reacted against Baber protein antigen of *S.paratyphi A*.

Our previously established mAb SPA20 which was very specific to S.paratyphi A (reported in progress report No. 4) was also tested in comparative immunoblotting between Barber protein antigen and cruded antigen extracted from fresh *S. paratyphi A* culture. The result revealed that its specific epitope was exactly the same 52 Kd protein band.

S.paratyphi B All 9 hybrids had IgG1K isotypes, they are shown in Table 2 The representative of this group (mAb 1C5D3) was used in the immunoblotting for confirmation of specificity and identification of molecular components of the specific antigen. The results of immunoblotting are shown in Fig. 2. The mAb 1C5D3 was very specific to *S.paratyphi B*

and the specific epitope which was recognized by this mAb was the protein which had a molecular weight of 52 Kd (Fig. 4). This 52 Kd protein band was also proved to be one of the prominent bands of *S.paratyphi B* protein antigen.

The specific epitope of our previously established SPB20 (reported in progress report No. 4) was also reconfirmed with comparative immunoblotting to be the protein band of 52 Kd molecular weight.

S.paratyphi C The 14 hybrids were divided into 4 groups according to their isotypes. Six clones had IgG1K isotype, 2 clones had IgG2bK isotype, 1 clone had IgG3K isotype and 5 clones had IgMK isotype. Data of the representative of each group is shown in Table 3. The immunoblotting against panel antigen is shown in Fig. 3. Both ELISA and immunoblotting results revealed that these mAbs were specific to *S.paratyphi C* but had cross reaction with *S.cholerasuis*. The mAbs 10B3E5 (IgG3K) and 10C2G6 (IgMK) were also specific to *S.paratyphi C* and *S.cholerasuis* in ELISA but showed no reaction with their antigens in immunoblotting technique, although we have repeated the experiment several times. The specific epitopes of *S. paratyphi C* (and probably *S.cholerasuis*) was protein which had molecular weight of 61 Kd (Fig. 4), and it was also proved to be one of the prominent bands of *S.paratyphi C* protein antigen.

S.typhi

Ten established mAbs which were very specific to *S. typhi* (reported in progress report No.6) have also been confirmed in the comparative immunoblotting to be specific to protein band of 52 Kd molecular weight (Fig. 4).

The 52 Kd protein band of *S.typhi*, *S.paratyphi A*, *S.paratyphi B* and 61 Kd protein band *S.paratyphi C* were proved to contain no carbohydrate by negative result when staining with concanavalin A. Hence, these findings suggested that our mAbs recognized pure amino acid molecules.

It is very interesting that the specific epitopes of *S.typhi*, *S.paratyphi A*, *S.paratyphi B* are protein and all have the same molecular weight of 52 Kd. This 52 Kd protein is noted to be one of the prominent protein antigens of Salmonellae.

OBJECTIVE II : To establish an ELISA test kit for the diagnosis of enteric fever.

We have performed biotin-avidin ELISA which we hoped that this system might amplify the sensitivity for antigen detection. The detail of biotin-avidin ELISA was as follows:

Each well of the alternated duplicated columns of Dynatech Immulon I ® Microelisa plate was coated with 5 μg/ml rabbit Ig anti-veronal buffer extract (VBE) of *S.typhi* in carbonate buffer. Five μg/ml of normal rabbit Ig in carbonate buffer was used to coat the remaining wells, for rule out false positivity or non-specific reactivity. The plate was incubated at 37°C for 3 hr, then washed with PBS-0.5% Tween 20 (PBST) and tapping dried. The plates were then blocked with 20% fetal calf serum (FCS) in PBS, incubated at 37°C for 1 hr and washed with PBST. One hundred microlitres of Bp antigen of *S.typhi* at various concentrations (in 20% FCS in PBS) was added to the plate and incubated at 4°C overnight. Each Bp antigen concentration was assayed in duplicate against both specific antibody and normal rabbit Ig. After 3 washes with PBST 10 μg/ml of mAb 2C1E10 in 20% FCS/PBS was added into the wells and the plate was incubated at 37°C for 1 hr, then washed. One hundred microlitres of 1:10,000 dilution of goat-antimouse immunoglobulin (Sigma) in 20% FCS/PBS was then added, incubated at 37°C for 1 hr and washed. Then one hundred microlitres of 1:10,000 dilution of biotinylated anti-goat immunoglobulin (Sigma) in PBS-0.5% Tween was added to the wells, incubated at 37°C for 1 hr and washed. One hundred microlitres of 1:2,000 dilution of Strepavidin-peroxidase conjugate (Sigma) in PBS-0.5% Tween was then added, incubated at 37°C for 1 hr. The excess conjugate was washed out 3 times with PBST and 100 μl of

substrate solution, [0.66 mg/ml 1,2-phenylene diamine dihydrochloride (Dakopatts) dissolved in 0.1 M citric acid phosphate buffer, pH 5.0, containing 0.0125% H_2O_2] was added to each well. The reaction was allowed to take place at 37°C for 1.hr and stopped by the addition of 4 M H_2SO_4 50 µl. The absorbance value was read spectrophotometrically at 492 nm (Titertek Multiskan ®, Flow laboratory, Bonn, West Germany). Several assays were performed with this mAb. In each assay, negative control, conjutate control and substrate blank were also included. The sample which showed positive result in the wells coated with specific antibody should showed negative reading in those coated with normal rabbit Ig.

The result is shown in Fig. 5 that this assay could detect approximately 0.05 µg/ml (50 ng/ml) of Barber protein antigen of *S.typhi* which was comparable to our newly established modified double antibody sandwich ELISA which was as follows:

The procedure was the same as biotin-avidin ELISA except 100 µl of 1:250 dilution of rabbit anti-mouse immunologulin-alkaline phosphatase conjugate (Dakopatts) in PBS-20% FCS was used in stead of the combined goat-antimouse Ig, biotinylated anti-goat Ig and Strepavidin-peroxidase conjugate. The substrate was p-nitrophenyl phosplate and the absorbance value was read at 405 nm. Each sample was performed in duplicate. The negative control, conjugate control and substrate blank were included in each assay. The sample which had positive reading with specific antibody should had negative reading in the wells coated with normal rabbit Ig. The result is shown in Fig. 6 that modified double antibody sandwich ELISA could detect approximately 0.05 µg/ml of *S.typhi* Bp antigen.

When we compared the biotin-avidin ELISA (B-A ELISA) with modified double antibody sandwich ELISA (MDA-ELISA). We found that, with the same total time requiring for the assay, both types of ELISA had the same sensitivity. Moreover, the MDA-ELISA had more advantage over B-A ELISA for its lower cost. Hence, we decided to use MDA-ELISA as our standard test for clinical specimens examination.

CLINICAL SPECIMENS TESTING FOR *S.TYPHI* ANTIGEN

We have used pooled mAbs which were specific to protein antigen of *S.typhi* namely mAbs 2C1G11 (IgG1K), 2B4D6 (IgG2bK), 5B6E3 (IgG1K) and 2C1E10 (IgG1K) in the MDA-ELISA for testing the following specimens. The positive control, negative control, conjugate control substrate blank as well as the non-specific control wells for rule out false positivity were all included in this assay.

Acute sera (obtained in the first day of admission)

S.typhi	8	specimens
S.paratyphi A	7	,,
Salmonella gr. B	19	,,
Salmonella gr. C	8	,,
Salmonella gr. D	1	,,
Other gm negative baeilli	13	,,
Gm positive cocci	3	,,

Urine samples (obtained in the 1st or 10th day of admission)

S.typhi	63	specimens	From the 1st day of admission
S.paratyphi A	8	,,	
Salmonella gr. B	26	,,	
Salmonella gr. C	7	,,	
Salmonella gr. D	1	,,	
Other gm negative baeilli	13	,,	
Gm positive cocci	3	,,	
Fever of undetermined origin (no hemoculture performed)	32	,,	

S.typhi	55	specimens	From the 10th day of admission
S.paratyphi A	1	,,	
Salmonella gr. B	8	,,	
Fever of undetermined origin (no hemoculture performed)	32	,,	

RESULTS AND DISCUSSION

With our MDA-ELISA, we have obtained true negative results from all sera and urine specimens. The data suggested that, our mAbs are very specific to *S.typhi* since no positive reaction was seen in the specimens from other enterobacteriaceae or gram positive cocci. The unability of these mAbs to detect the antigens in the clinical specimens might be due to the fact that these mAbs can see only their

specific epitopes (52 Kd protein) but this 52 Kd protein are not present in the blood and urine of the patient or present in a very minute amount beyond the sensitivity of our test system.

Although in our hands, our mAbs can not be used for *S.typhi* antigen detection in the clinical specimen of the patients, these valuable mAbs can be very useful for future researches. They are now being tested in our laboratory for their efficiency in detecting the antigens in hemoculture with the hope that such a procedure might shortened the time requiring for bacterial laboratory diagnosis of typhoid fever. They can be used for biochemical or biotechnological purification of *S.typhi* 52 Kd protein and this unique antigen will be very useful for serodiagnosis of typhoid fever by detecting specific IgM in acute serum of typhoid patients. Moreover, this specific antigen can be used as a tool for various immunological researches for better understanding of immunopathogenesis of this disease as well as studying Salmonella taxonomy.

WORKPLANS FOR THE NEXT PERIOD

I. Clinical specimens testing by our established mAbs (the one that specific to *S.paratyphi A* or *S.paratyphi B* or *S.paratyphi C*).

II. Establishment of ELISA test kits, if the favourable result is obtained from I.

Table 1. Representative of mAbs specific to *S.paratyphi A*, one from each groups, demonstrate isotype, molecular weight (M.W.) of specific protein epitopes and reactivities to a panel of Barber protein extracted from 12 bacterial strains in ELISA. The cut off O.D. value is 0.20.

				O.D. against cruded protein antigen of											
Group	Clone name	Isotype	M.W. (Kd)	S. typhi	S. paratyphi A	S. paratyphi B	S. paratyphi C	S. cholerasuis	S. enteritidis	S. krefeld	S. panama	S. typhimurium	E. coli	Ps. pseudomallei	Y. enterocolitica
I	1C3G11	IgG1K	52	0.11	>2	0.12	0.13	0.11	0.12	0.11	0.12	0.12	0.13	0.11	0.15
II	4A6D10	IgG2bK	52	0.13	>2	0.13	0.11	0.16	0.15	0.13	0.13	0.12	0.16	0.19	0.11
III	8D6E1	IgG2bK	52	0.14	>2	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.16	0.14	0.15
IV	7B2G3	IgG3K	52	0.15	1.7	0.17	0.14	0.14	0.14	0.16	0.14	0.16	0.13	0.15	0.13

epitopes and reactivities to a panel of Barber protein extracted from 12 bacterial strains in ELISA. The cut off O.D. value is 0.20.

Clone name	Isotype	M.W. (Kd)	O.D. against cruded protein antigen of											
			S. typhi	S. paratyphi A	S. paratyphi B	S. paratyphi C	S. cholerasuis	S. enteritidis	S. krefeld	S. panama	S. typhimurium	E. coli	Ps. pseudomallei	Y. enterocolitica
1A5G9	IgG1K	52	0.12	0.14	0.6	0.11	0.17	0.15	0.13	0.11	0.13	0.16	0.15	0.15
1C1G2	,,	,,	0.14	0.12	0.9	0.12	0.14	0.13	0.13	0.12	0.13	0.13	0.14	0.14
1C5D3	,,	,,	0.12	0.13	1.6	0.12	0.14	0.12	0.13	0.11	0.12	0.16	0.14	0.15
1C5F2	,,	,,	0.12	0.18	1.6	0.11	0.15	0.13	0.12	0.13	0.12	0.16	0.14	0.15
1C5F4	,,	,,	0.18	0.17	1.6	0.15	0.18	0.18	0.14	0.16	0.16	0.17	0.17	0.17
1C6F9	,,	,,	0.11	0.12	1.6	0.13	0.12	0.12	0.13	0.11	0.11	0.12	0.13	0.12
5B3D5	,,	,,	0.15	0.12	1.0	0.12	0.14	0.13	0.14	0.13	0.13	0.14	0.14	0.14
8B2H3	,,	,,	0.13	0.17	1.2	0.14	0.12	0.12	0.14	0.12	0.13	0.11	0.13	0.11
9B2G8	,,	,,	0.12	0.17	1.9	0.13	0.12	0.11	0.13	0.12	0.15	0.11	0.14	0.12

Table 3. Representative of mAbs specific to *S.paratyphi C*, one from each groups, demonstrate isotype, molecular weight (M.W.) of specific protein epitopes and reactivities to a panel of Barber protein extracted from 12 bacterial strains in ELISA. The cut off O.D. value is 0.20.

				O.D. against cruded protein antigen of											
Group	Clone name	Isotype	M.W. (Kd)	*S. typhi*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*	*S. cholerasuis*	*S. enteritidis*	*S. krefeld*	*S. panama*	*S. typhimurium*	*E. coli*	*Ps. pseudomallei*	*Y. enterocolitica*
I	1B2F10	IgG1K	52	0.14	0.14	0.15	0.8	0.98	0.16	0.16	0.14	0.16	0.13	0.15	0.13
II	2B4E10	IgG2bK	52	0.13	0.12	0.12	0.5	0.91	0.12	0.12	0.12	0.12	0.12	0.12	0.11
III	10B3E5	IgG3K	-	0.14	0.13	0.13	1.9	1.9	0.15	0.14	0.14	0.15	0.14	0.18	0.13
IV	10C2G6	IgMK	-	0.15	0.13	0.12	1.8	1.8	0.14	0.14	0.14	0.13	0.12	0.14	0.11

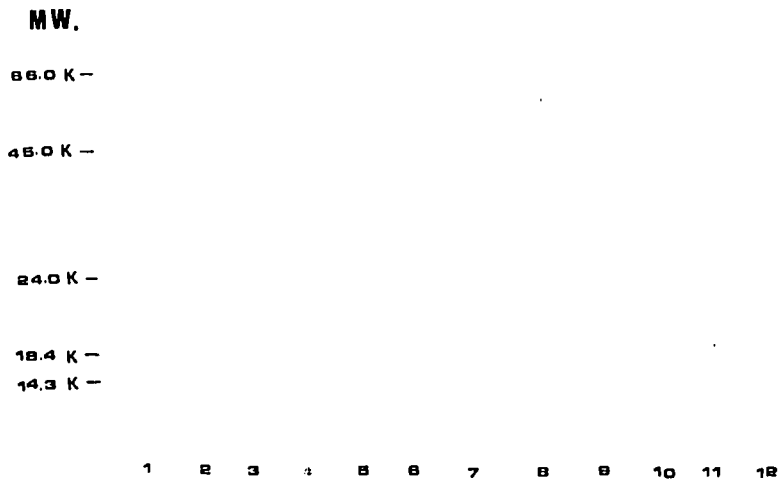

Fig. 1 Immunoblotting analysis of the Bp antigen from *S. paratyphi A* (lane 1), *S.typhi* (lane 2), *S.paratyphi B* (lane 3), *S.paratyphi C* (lane 4), *S.cholerasuis* (lane 5), *S.enteritidis* (lane 6), *S.krefeld* (lane 7), *S. panama* (lane 8), *S.typhimurium* (lane 9), *E.coli* (lane 10), *Ps.pseudomallei* (lane 11), and *Y.enterocolitica* (lane 12). Each antigen was run in SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with mAb 1C3G11. Alkaline phosphatase-labeled goat-anti mouse immunoglobulin was used as a second antibody. The result revealed specificity of mAb 1C3G11 to *S. paratyphi A*.

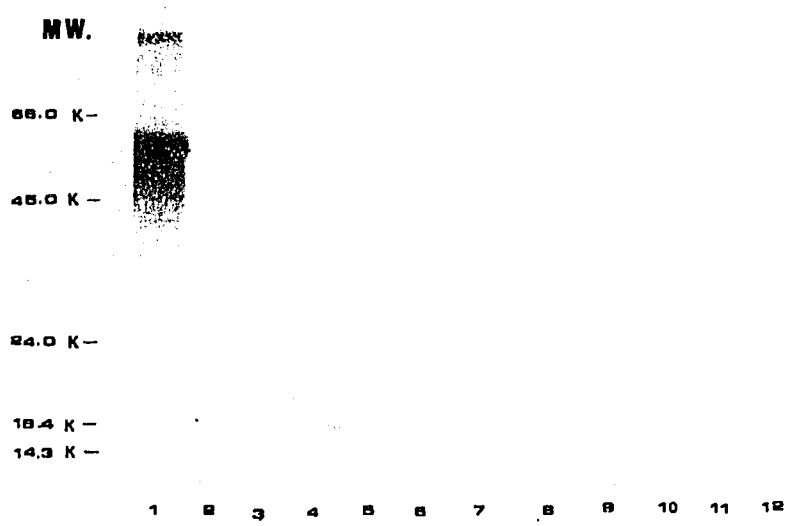

Fig. 2 Immunoblotting analysis of the Bp antigen from *S. paratyphi B* (lane 1), *S.typhi* (lane 2), *S.paratyphi A* (lane 3), *S.paratyphi C* (lane 4), *S.cholerasuis* (lane 5), *S.enteritidis* (lane 6), *S.krefeld* (lane 7), *S. panama* (lane 8), *S.typhimurium* (lane 9), *E.coli* (lane 10), *Ps.pseudomallei* (lane 11), and *Y.enterocolitica* (lane 12). Each antigen was run in SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with mAb 1C5D3. Alkaline phosphatase-labeled goat-anti mouse immunoglobulin was used as a second antibody. The result revealed specificity of mAb 1C5D3 to *S. paratyphi B*.

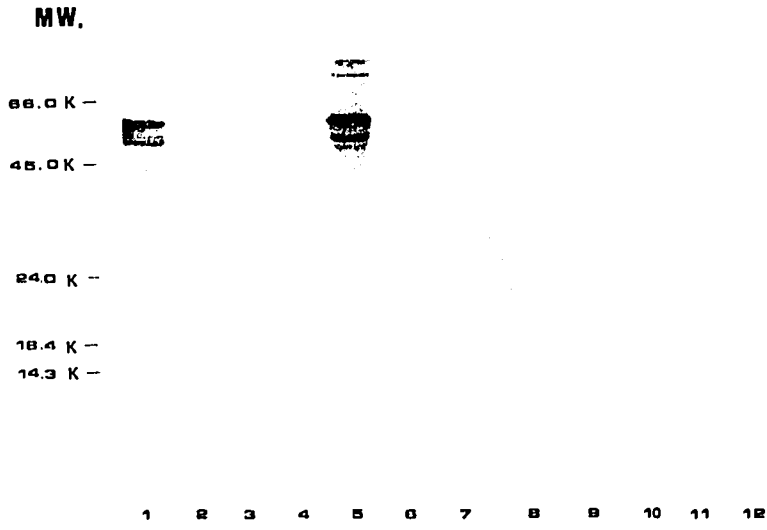

Fig. 3 Immunoblotting analysis of the Bp antigen from *S. paratyphi C* (lane 1), *S.typhi* (lane 2), *S.paratyphi A* (lane 3), *S.paratyphi B* (lane 4), *S.cholerasuis* (lane 5), *S.enteritidis* (lane 6), *S.krefeld* (lane 7), *S. panama* (lane 8), *S.typhimurium* (lane 9), *E.coli* (lane 10), *Ps.pseudomallei* (lane 11), and *Y.enterocolitica* (lane 12). Each antigen was run in SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with mAb 1B2F10. Alkaline phosphatase-labeled goat-anti mouse immunoglobulin was used as a second antibody. The result revealed specificity of mAb 1B2F10 to *S. paratyphi C*.

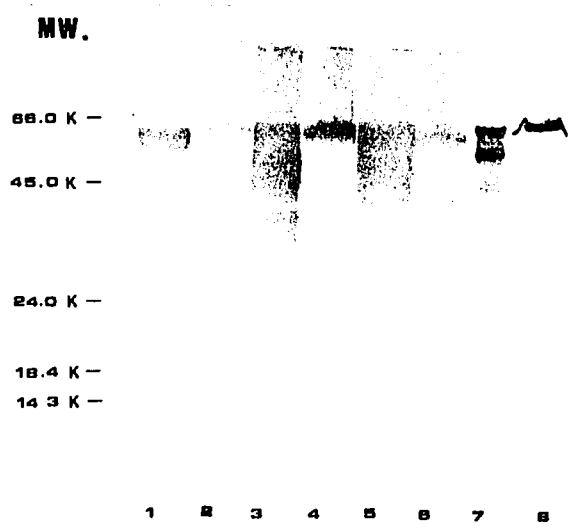

Fig. 4 Comparative immunoblotting analysis between the Bp antigen of *S.typhi* (lane 1) and cruded antigen extracted from boiling fresh *S.typhi* (lane 2), the Bp (lane 3) and cruded antigen extracted (lane 4) of *S. paratyphi A*, the Bp (lane 5) and cruded antigen extracted (lane 6) of *S.paratyphi B*, the Bp (lane 7) and cruded antigen extracted (lane 8) of *S.paratyphi C*. Each antigen was run in SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with their corresponding mAb. Alkaline phosplatase-labeled goat-anti mouse immunoglobulin was used as a second antibody. The specific antigen of all 3 Salmonellae have the same molecular weight of 52 Kd, except *S. paratyphi C* which has 61 Kd molecular weight specific epitopes.

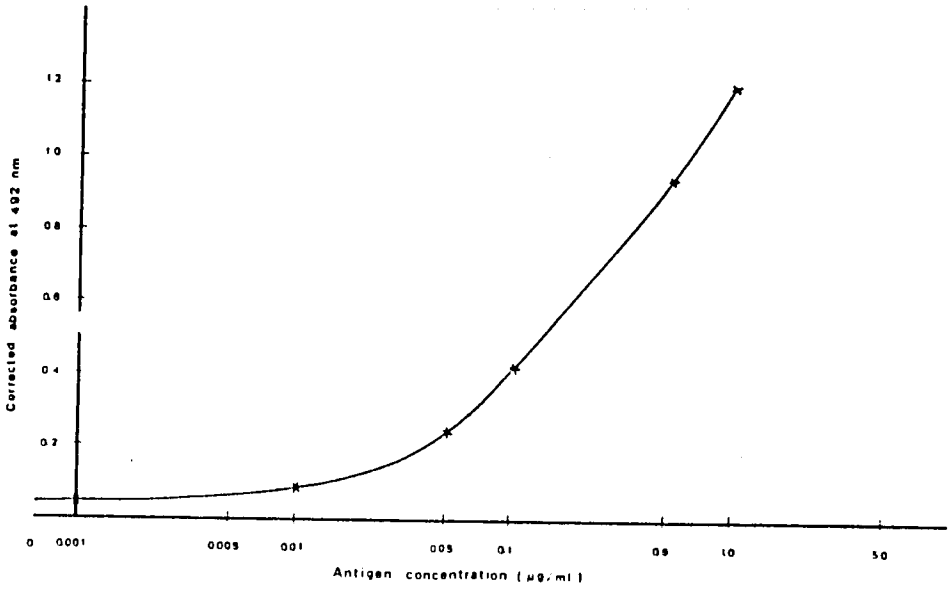

Fig. 5 Dose response curves obtained from the assay of Barber protein antigen of *S. typhi* at various concentration with biotin-avidin ELISA, using goat anti-mouse Ig 1:10,000 in 20% FCS in PBS, This assay could detect approximabty 0.05 µg/ml antigen.

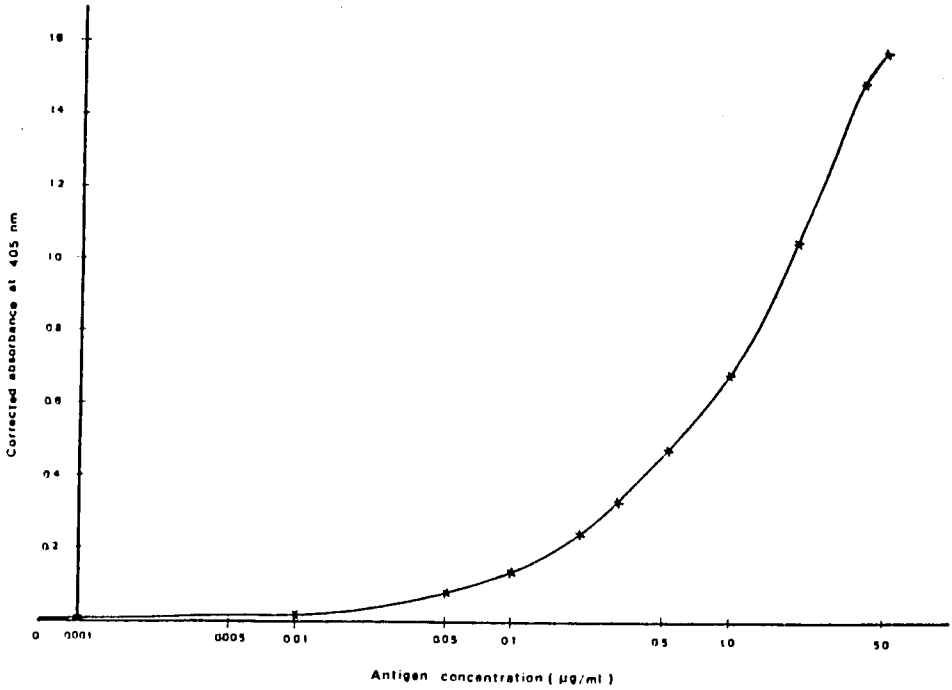

Fig. 6 Dose response curve obtained from the assay of Barber protein antigen of *S.typhi* at various concentration with modified double antibody sandwich ELISA. This assay could detect approximately 0.05 µg/ml antigen.