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ORIGINAL ARTICLES

Gel-filtration Chromatography of *Salmonella* Protein Antigens and Their Implication in Immunodiagnosis

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SUMMARY **Crude Barber protein (Bp) antigens were prepared from *Salmonella typhi, S. krefeld* and *S. derby* by an original method that has been described previously. These antigens were subjected to gel – filtration chromatography using Sephadex G – 200. A sharp peak that eluted together with the void volume was thus separated from a broad second peak that eluted from the column at positions equivalent to 118,000 to 12,000 daltons. The proteins eluted in the latter peak were arbitrarily divided into 5 fractions and, together with the first peak, subjected to polyacrylamide gel electrophoresis and immunoprecipitation with both homologous and heterologous rabbit antisera. The extent of immunological cross reactivities was determined by enzyme – linked immunosorbent assay. The preliminary results obtained by this technique showed species – specific protein antigens to have molecular weights ranging between 36,000 and 68,000 daltons.**

Cross reactivities among the antigens of *Salmonella,* non-*Salmonella* Enterobacteriaceae and other gram-negative bacilli have been well documented. For instance, Espersen *et al.*[1] found as many as 86 different antigens in sonicated *S. typhi* preparations, many of which exhibited intense cross reaction with antigens from other Enterobacteriaceae. A less extensive but significant cross reaction between antigens from *S. typhi* and other gram-negative bacilli not in the Enterobacteriaceae group has also been observed, but under the same conditions, no cross reaction with gram-positive bacteria could be detected.[2] Attempts to eliminate cross reactivities by absorption have been unsuccessful.[3] Hence, immunodiagnosis using such antigens for the detection of a specific antibody employing only a single serum specimen has not been possible. In order to overcome these problems, we partially purified *Salmonella* protein antigen from crude Barber protein preparations by gel-filtration chromatography using Sephadex G-200. The preliminary results presented in this communication showed that it was possible to eliminate a considerable degree of immunological cross reactivity among the 3 *Salmonella* species used by this simple purification technique.

MATERIALS AND METHODS

Preparation of Barber proteins

Barber proteins (Bp) were prepared from *S. typhi* 0901, *S. krefeld* and *S. derby* essentially as originally described by Barber, Vladoianu and Dimache.[4] The crude Barber protein solution obtained was sterilized by filtration prior to being lyophilized and stored at 4°C.

Preparation of rabbit antisera to crude Barber proteins

Rabbit antisera to Barber protein from each of the three *Salmonella* species were prepared as described elsewhere.[5] Briefly, an adult albino rabbit weighing approximately 2 kg was immunized in the two front foot pads and 4 other subcutaneous sites with 2 mg of protein homogenized in complete Freund's adjuvant. One month later, the animal was given three booster injections every other day, one each by the subcutaneous route, the intra – muscular route and the intravenous route. Each consisted of 1 mg Barber protein in 0.85% NaCl (saline). The animal was bled 7 days after the last injection. Blood was allowed to

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clot and the serum was separated and stored at −20°C.

Enzyme – linked immunosorbent assay (ELISA)

The interaction between anti-Barber protein sera with crude or fractionated Bp was investigated by indirect ELISA.[6] An optimal antigen concentration was found to be 20 μg/ml by a checker-board titration. Antibody activity was expressed using either the positive to negative (P/N) ratio method[7] or the end-point method.[8] Antibody titers for the latter were calculated using as the end point the absorbance value of 0.4 above that of the background.

Other techniques

Radioimmunoprecipitation. The Bp components reactive with homologous and heterologous rabbit antisera were analyzed by radio-immunoprecipitation and poly-acrylamide gel electrophoresis essentially as described previously by Wongratanacheewin *et al.*[9] Molecular weights of reactive components were subsequently determined.[10]

Protein determination. Protein concentration was determined by the Folin method,[11] using bovine serum albumin as a standard.

RESULTS

Complexity of crude *Salmonella* Barber proteins

The complexity of crude Bp extracted from *S. typhi, S. krefeld* and *S. derby* was analyzed by SDS-PAGE and radioimmunoprecipitation. The crude preparations from all three species of *Salmonella* exhibited similar staining patterns regardless of whether the electrophoresis was carried out under reduced or un-reduced conditions (Fig. 1). There were at least 30 protein compoments with relative molecular weights that varied from 12,000 to > 80,000 daltons. For all 3 species, the most intensely stained protein component had M_r of 31,000 daltons. With the exception of a low molecular weight doublet (M_r = 13,000) which took up carbohydrate staining faintly, all other components appeared to be devoid of a carbohydrate moiety. The species differences that could be readily observed were differences in the relative intensities of individual components.

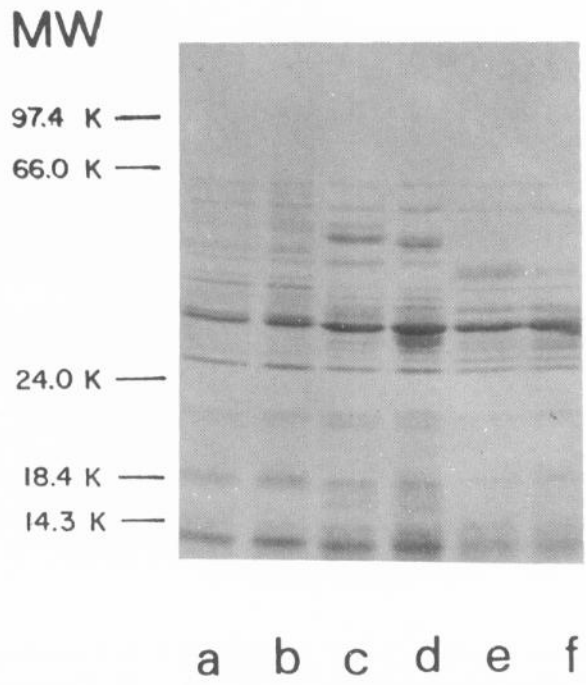

Fig. 1 SDS – PAGE protein (stained with Coomassie blue) profiles of reduced (a, c and e) and unreduced (b, d and f) crude Barber proteins prepared from *S. typhi* (a, b), *S. derby* (c, d) and *S. krefeld* (e, f).

Autoradiography of the immunoprecipitate of iodine-labeled crude Bp and homologous antiserum was also complex and as many as half of the Coomassie blue stained protein components could be readily detected. The components that reacted most strongly were those having M_r of 13,000 to 15,000 and of 25,000 to 40,000. An example of the interaction of *S. typhi* Bp with the homologous anti-*S. typhi* and the heterologous anti-*S. krefeld* and *S. derby* sera is shown in Figure 2. A strong immunological cross-reactivity among the three crude Barber protein preparations is supported by ELISA titration curves of these crude antigens with the three rabbit antisera (Figs. 3-5) and by the calculated P/N ratios (Table 1).

Because the crude Barber proteins extracted from these three species of *Salmonella* are physico-chemically and immunologically complicated, their usefuless in immunodiagnosis is limited. An attempt was therefore made to minimize immunological cross-reactivity, using these 3 species as models. An initial attempt involved partial separation of the crude Bp preparation by gel-filtration chro-matography using Sephadex G-200. The elution patterns of all three

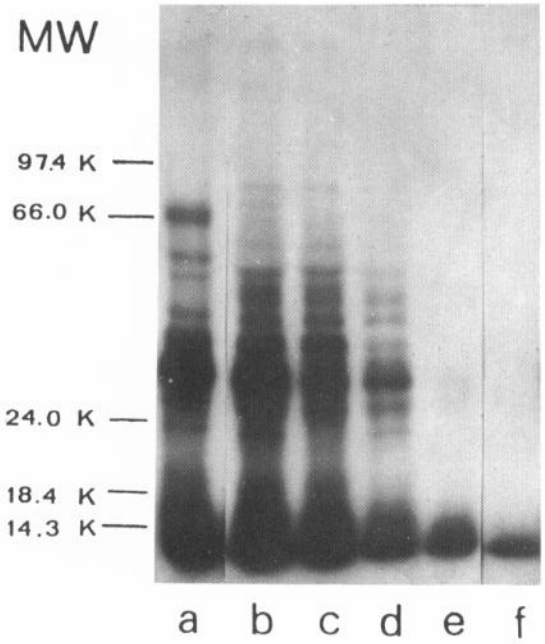

Fig. 2 Autoradiograms of SDS – PAGE patterns of ^{125}I – labelled *S. typhi Bp (a) and of labelled Bp immunoprecipitated with rabbit anti – S. typhi* Bp (b), rabbit anti – *S. derby* Bp (c), rabbit anti – S. *krefeld* (d), normal rabbit serum (e) and protein – A Sepharose (f).

crude Bp preparations were similar. An example of such a pattern is shown in Figure 6 for *S. typhi*. A sharp peak that eluted together with the void volume could be separated from the second broad peak that eluted from the column at positions 12,000 to 118,000 daltons. Subsequently, the second peak was divided arbitrarily into 5 fractions as indicated in the Figure. SDS-PAGE analysis showed each of these fractions to be considerably less complicated than the crude *S. typhi* Bp although they were still rather heterogeneous by radioimmunoprecipitation.

Table 1 Positive/negative (P/N) ratios of rabbit anti – *Salmonella* Bp sera

S. typhi Bp antigens	P/N ratio at 1:15625*		
	Anti-*S. typhi* BP	Anti-*S. krefeld* BP	Anti-*S. derby* BP
Unfractionated	61	38	45
Fractionated+			
F1	28	19	23
F2	46	5	4
F3	55	3	2
F4	30	3	3
F5	32	4	5

* Optical – density ratios at a serum dilution of 1:15625
\+ Fractionated by gel – filtration chromatography using sephadex G – 200 (see Fig. 6).

The immunological reactivities of these partially purified fractions were evaluated by ELISA using both homologous and heterologous antisera. The ELISA patterns of unfractionated (Fig. 7) and fractionated (Figs. 8-12) *S. typhi* Bp fractions showed marked differences in reactivities after fractionation. Results shown in these figures and summarized in Table 1 demonstrated that a simple gel-filtration procedure was rather effective in minimizing cross-reactivity among the Bp from *Salmonella* species. The more species-specific antigen resided largely in Fraction 3 (F3), as shown by the ELISA titration patterns in Figure 10 and a marked reduction in the P/N ratios obtained with the heterologous antisera (Table 1). The components eluted from the column at the F3 position would have calculated molecular weights of 36,000-68,000 daltons. The components eluted from the column together with the void volume (F1) seemed to contain largely *Salmonella* common antigens. Both the ELISA titration curves (Fig. 8) and the P/N ratios against this high molecular weight fraction (Table 1) were similar to those exhibited by unfractionated crude *S. typhi* Bp (Fig. 7).

Similar results were obtained when crude *S. krefeld* and *S. derby* Barber proteins were fractionated and tested as described herein for *S. typhi* BP. Again, the more species-specific antigens appeared to have molecular weights ranging between 20,000 and 50,000 daltons.

DISCUSSION

It is well documented that immunological cross reactivities occur among Enterobacteria species and other gram-negative bacilli.[2,3] Immunodiagnosis based on the detection of specific antibody and/or antigen in specimens from suspected individuals is largely unsatisfactory. A rising antibody titer in the paired

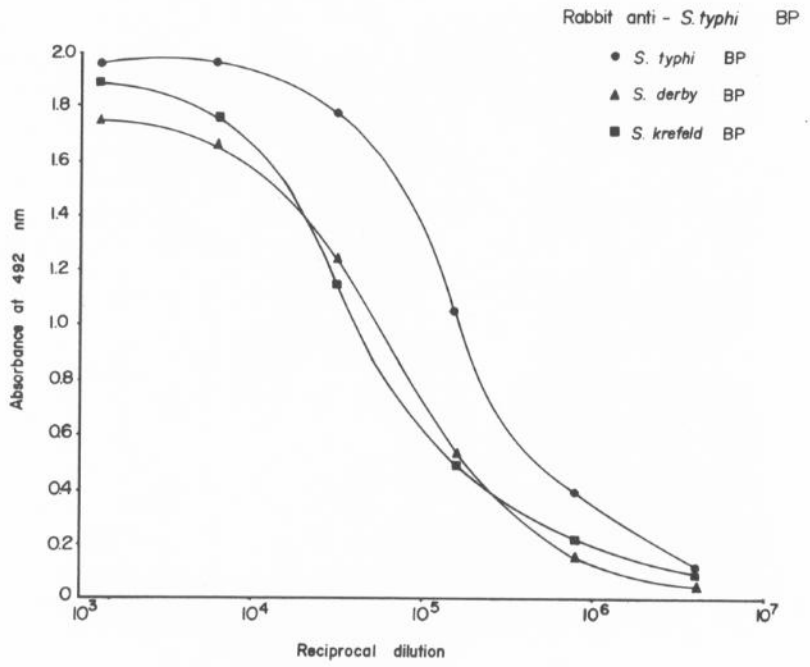

Fig. 3 ELISA titration curves of rabbit anti – *S. typhi* Bp reacted with homologous. *(S. typhi)* and heterologous *(S. derby* and *S.krefeld) Barber proteins.*

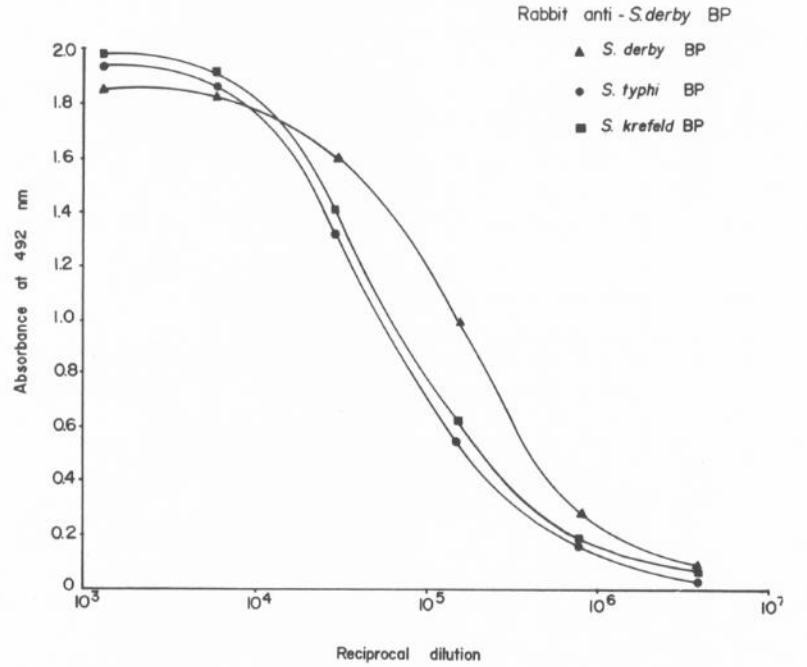

Fig. 4 ELISA titration curves of rabbit anti – *S. derby* Bp reacted with homologous *(S. derby)* and heterologous *(S. typhi* and *S. krefeld)* Barber proteins.

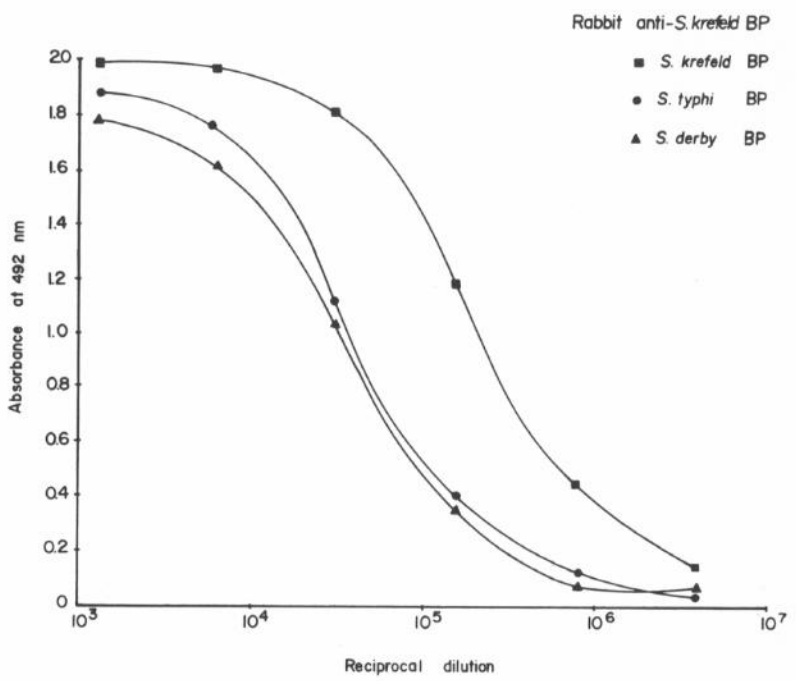

Fig. 5 ELISA titration curves of rabbit anti – *S. krefeld* Bp reacted with homologous *(S. krefeld)* and heterologous *(S. typhi* and *S. derby)* Barber proteins.

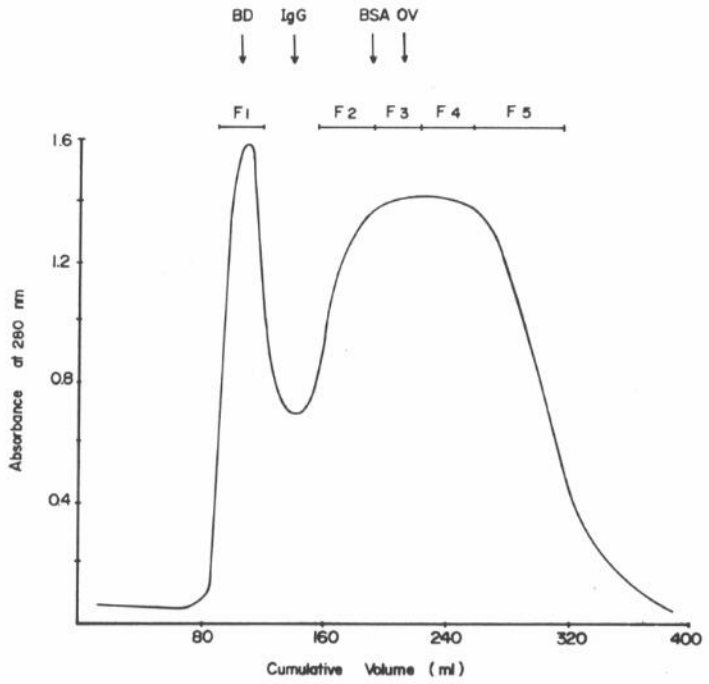

Fig. 6 Chromatographic pattern of crude *S. typhi* Bp (60 mg) eluted from a Sephadex G – 200 column (2.5 × 65 cm) with a 0.15 M potassium phosphate buffer pH 7.2. Five – ml fractions, collected at a constant flow rate of 15 ml/hr, were pooled into 5 portions as indicated. BD (blue dextran), IgG (immunogloulin G), BSA (bovine serum albumin) and OV (ovalbumin) were used as standard markers.

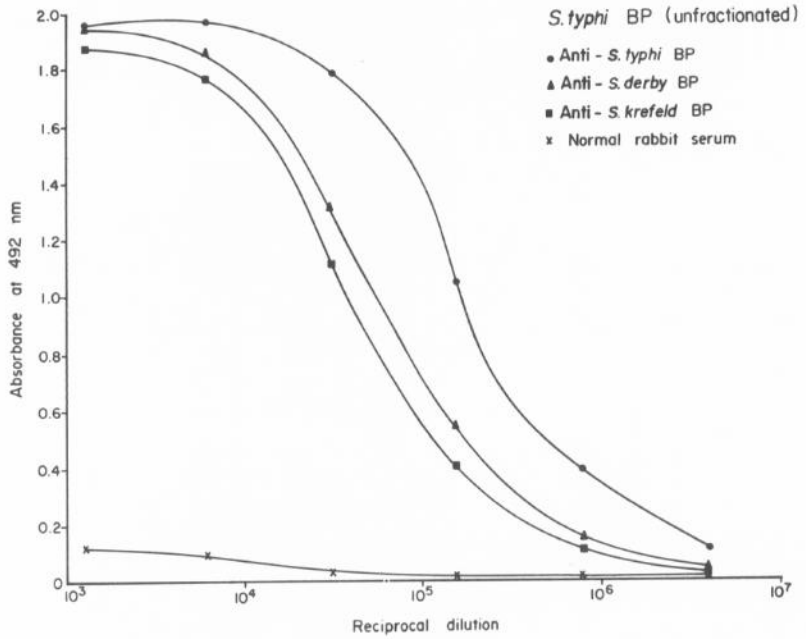

Fig. 7 ELISA titration curves of rabbit anti – *S. typhi* Bp, rabbit anti – *S. derby* Bp and rabbit anti – *S. krefeld* Bp with unfractionated crude *S. typhi* Bp.

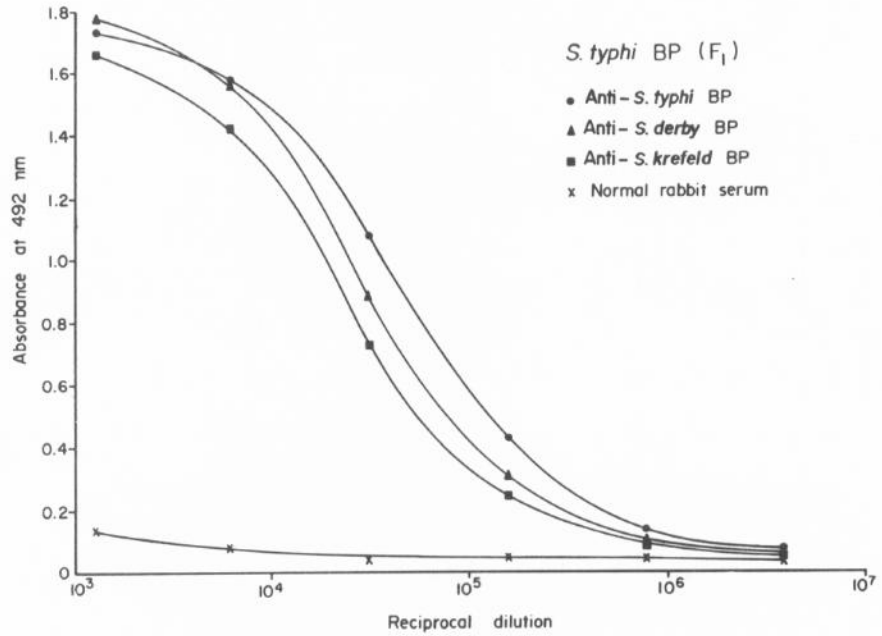

Fig. 8 ELISA titration curves of rabbit anti – *S. typhi* Bp, rabbit anti – *S. derby* Bp and rabbit anti – *S. krefeld* Bp with the *S. typhi* Bp fraction eluted together with the void volume (M_r > 200,000 daltons).

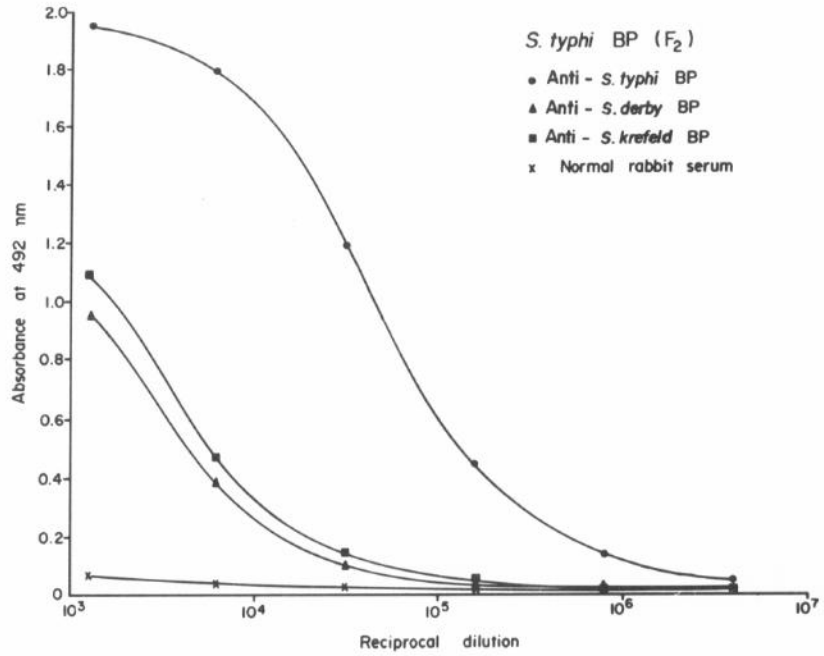

Fig. 9 ELISA titration curves of rabbit anti – *S. typhi* Bp, rabbit anti – *S. derby* Bp and rabbit anti – *S. krefeld* Bp with the *S. typhi* Bp fraction (F2) eluted from the Sephadex column at M_r 118,000 – 69,000 daltons.

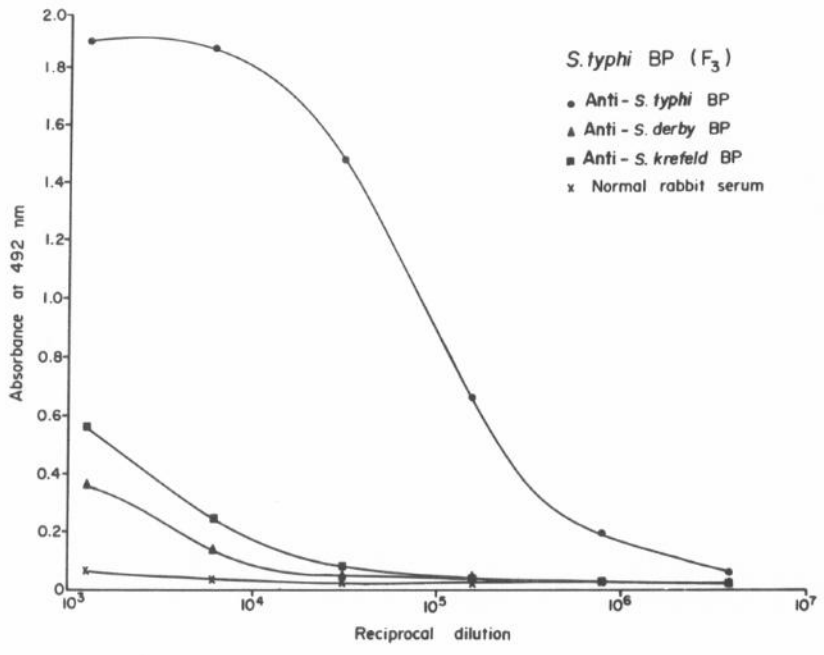

Fig. 10 ELISA titration curves of rabbit anti – *S. typhi* Bp, rabbit anti – *S. derby* Bp and rabbit anti – *S. krefeld* Bp with the *S. typhi* Bp fraction (F3) eluted from the Sephadex column at M_r 68,000 – 36,000 daltons.

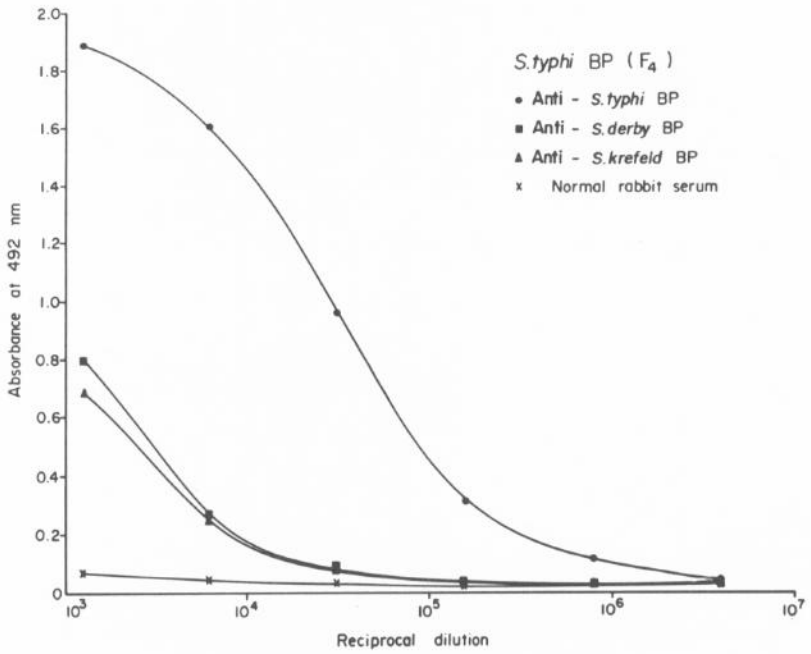

Fig. 11 ELISA titration curves of rabbit anti – *S. typhi* Bp, rabbit anti – *S. derby* Bp and rabbit anti – *S. krefeld* Bp with the *S. typhi* Bp fraction (F4) eluted from the Sephadex column at M_r 35,000 – 18,500 daltons.

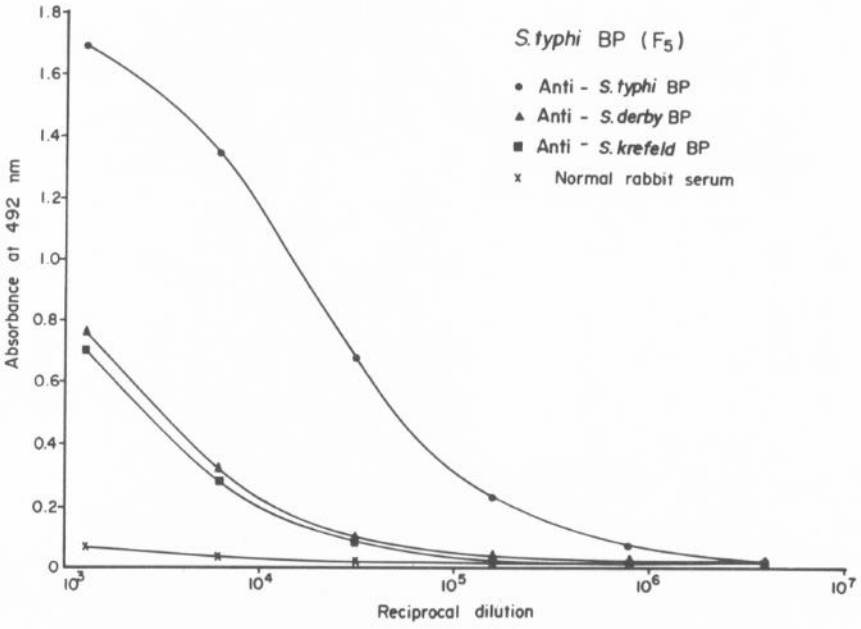

Fig. 12 ELISA titration curves of rabbit anti – *S. typhi* Bp, rabbit anti – *S. derby* Bp and rabbit anti – *S. krefeld* Bp with the *S. typhi* Bp fraction (F5) eluted from the Sephadex column at M_r < 17,000 daltons.

serum from these individuals is generally accepted as a reliable indicator for active infection.[12] However, when only a single serum specimen is available, such a conclusion cannot be made. In the past, attempts had been made to improve on the specificity of the test system by using different extraction procedures and other classical approaches, but these attempts have been unsuccessful. Several groups of investigators have used crude Barber protein preparations but failed to obtain the degree of specificity required. The cross reactivities noted among different *Salmonella* species are not unexpected and this is clearly documented by the data presented in this study, using rabbit antisera against different Bp preparations (Figs. 3-5). For example, results presented in Figure 1 and summarized in Table 1 clearly showed that antibody to *S. typhi* reacted almost equally well with the heterologous Bp from *S. krefeld* and *S. derby* when compared with the homologous *S. typhi* Bp. However, a significant improvement was obtained when these crude preparations were subjected to a simple single-step purification using a gel-filtration chromatography technique.

The ELISA results with the various Sephadex G-200 fractions (Fig. 6) demonstrated clearly that it was possible to eliminate a considerable proportion of immunological cross reactivity among the 3 *Salmonella* Bp by gel-filtration chromatography, as the crossreactive components appeared to elute from the column together with the void volume (Fit. 8).

Because the remaining components eluted from the column as one broad peak, we had to arbitrarily divide and pool them into the subfractions shown in Fig. 6. The immunological reactivity profiles of these various fractions were similar among the 3 *Salmonella* species used. For example, the relatively more species-specific antigens were present largely in fractions having relative molecular weights between 18,500 and 68,000, when the calculation was based on their eluted positions from the column. On the other hand, those components with lower relative molecular weights appeared to be less immunogenic. Moreover, they seemed to cross react more extensively than the larger components eluted immediately ahead.

Although the number of *Salmonella* species tested was limited, our preliminary results clearly demonstrated that it is possible to purify *Salmonella* species – specific antigens by a simple physicochemical technique. We have recently observed that a combination of gel filtration and affinity column chromatography gave a significant improvement (unpublished observations). However, because the yield obtained from the latter procedure was very low, modification has to be made if one is to mass produce these antigens for diagnostic purposes. With the new technologies currently available, success should not be too far away, particularly after the antigens have been identified and characterized.

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