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Immunogenicity and specificity of *Salmonella typhimurium* outer membrane antigens

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Abstract

S. typhimurium was acetone dried, negative stained, and its outer membrane was evaluated by transmission electron microscopy. White Albino rabbits were immunized against acetone derived *S. typhimurium*, and the immune sera were collected. Outer membrane antigens were extracted from acetone dried and veronal buffer treated *S. typhimurium*, *S. typhi*, *S. paratyphi-B*, *E. coli*, and *S. flexineri*. *S. typhimurium* extract was further fractionated. Immunogenicity and cross reactivity of each antigenic fraction were evaluated by ELISA and immunodiffusion using the immune sera and commercially available or in-house product sera. Molecular sizes of proteins in fractions were evaluated using SDS-PAGE. A *S. typhimurium* specific 70 kDa antigen was detected. However, highest antigenicity was detected in 36-43 kDa common protein fraction. These antigenic fractions should have utility in medical microbial immunology.

Key words: *Salmonella typhimurium*, bacterial antigens, rabbit antibodies, outer membrane, Enterobacteriaceae

Salmonella typhimurium dış membran antijenlerinin immunojenite ve özgüllüğü

Özet

S. typhimurium asetonla kurutuldu, negatif boyandı ve transmisyon elektron mikroskopu ile dış membranı incelendi. Sağlıklı beyaz Albino tavşanlar asetonla kurutulmuş bakteri ile bağışıklandı ve bağışık serum toplandı. Asetonla kurutulmuş *S. typhimurium*, *S. typhi*, *S. paratyphi-B*, *E. coli*, ve *S. flexineri*'den veronal tamponla muamele edilerek dış membran antijenleri ekstrakte edildi. *S. typhimurium* ekstratı daha ileri fraksiyonlara ayrıldı. Hazırlanan bağışık serum ve firmalardan alınan veya laboratuvarda hazırlanmış bağışık serumlar kullanılarak her bir antijenik fraksiyonun immunojenite ve çapraz reaksiyonu ELISA ve immunodifuzyon yöntemleriyle incelendi. Fraksiyonlardaki proteinlerin moleküler büyüklüğü SDS-PAGE ile tayin edildi. *S. typhimurium*'a özgü 70 kDa büyüklüğünde bir antijen saptandı. Diğer yandan, 36-43 kDa ortak proteinler taşıyan fraksiyon en yüksek antijeniteyi gösterdi. Bu fraksiyonlar tıbbi mikrobiyal immunolojide kullanım alanı bulabilecektir.

Anahtar sözcükler: *Salmonella typhimurium*, bakteriyal antijenler, tavşan antikorları, dış membran, Enterobacteriaceae

Introduction

The incidence of *S. typhimurium* infections in recent years in Turkey was increased, and high child mortality due to gastrointestinal infections was detected (İçgen et al., 2002; Willke et al., 2002). Since common laboratory techniques does not support the needs of specialists for efficient treatment and follow up the cases, more accurate or complementary approaches started to evolve to fulfill the needs (Jongerius-Gortemaker et al., 2002; Bacarese-Hamilton et al., 2002; Prager et al., 2003). One of them is to detect specific bacterial markers in patient materials using immunochemical techniques (Akış et al., 1990; Skov et al., 2002). Although, the members of Enterobacteriaceae carry rich antigens and immunogens, and they are relatively easy to derive, isolation of specific components has been more difficult. Herein we report *S. typhmurium* specific and group specific protein markers, which should have utility in medical microbial immunology and diagnostic laboratories. The methodologies used to isolate membrane antigens should be useful for investigation in multiple areas.

Material and Methods

Microorganisms

Salmonella typhimurium (KEUN-886 (B) S1-28-8 "K form"); *Salmonella paratyphi-B* (KUEN 1256 S1-21-47 "H form"); *Salmonella typhi* (KUEN1253 (B) S1-27-74 "H form"); *E. coli* (ATCC 11,229, 21. 4. 1988) and *Shigella flexineri* (KUEN 1286 (B) S5-4-12, tip 2a) were provided from KUKENS (University of Istanbul, Istanbul, Turkey).

Electron microscopy

S. typhimurium was acetone-dried and examined as described (Akış et al., 1989).

Rabbits and sera

Female Wistar Albino and Black rabbits were purchased from Institute for Experimental Medical Research (University of İstanbul, Turkey). All animal experimentation described in the manuscript were conducted in accord with the Departmental Guide for the Care and Use of Laboratory Animals. In preparation of immune sera acetone dried *S. typhimurium* was sterilized using ethylene oxide and 0.2 mg of bacteria was suspended in 0.4 ml volume of serum physiologic. Rabbits were inoculated intradermal 17 times in four sessions with 2-6 weeks intervals. 2-6 immunization were applied in each session with 4-6 d intervals. Complete Freund's adjuvant was used in the first sessions' immunizations. The sites for injections were neck in the first and fourth sessions, and ventral lumber area in the others. Sera were collected with 3 d intervals after each session was completed, and were tested for specific antibody by immunodiffusion. The highest serum titers were detected after 4th session, and blood was collected from the ear vein of the animals. Sera were separated and aliquots were kept at -20°C (Çetin et al., 1973). Control sera were in-house product immune sera against *S. typhi* (O- and H-sera), *S. paratyphi-B* (O- and H-sera), *S. typhimurium* (H-sera), *Proteus* OX_2 and -OX_{19} which were prepared according to standard protocols (Kaufman et al., 1960) and polyclonal sera against *E. coli* 1-4 and against *Shigella* (mixture of *S. flexineri* and *S. dysenterie*) were purchased (Wellcome, Oxford, UK).

Immunodiffusion

2.5 mm thick 0.85% agarose gel (buffer: 0.24% TRIS; 0.15% Glycine; 0.29% EDTA; 0.01% NaN_3 (Merck, San Diego, CA); pH 7.2) on slides were used to open holes 0.2 mm in diameter at 4 mm distance by sucking. Sera and fra-TXY for all bacteries were loaded in holes in proximity. The slides were incubated in humidity chamber for 3 d, first day at 37°C, second day at room temperature, and last day in refrigerator. The precipitated immune complex bands in between holes were visualized under luminated light, and pictures were taken. None of the reagents were diluted except that LPS (Difco, Bruxelles, Belgium) was used at 5 mg/ml concentration based on the results of optimization study. Some results were confirmed by counterimmunodiffusion. In these experiments, 0.9% indubiose agarose gel (buffer: 1.05% sodium barburate; 0.17% barbital; 0.15% calcium lactate; pH 8.6) was used and preceded as described above. Antigens were loaded in holes at cathode pole and the electrophoresis was completed under 22 mA.

Antigens

Bacteria were cultured and acetone dried, and antigenic fractions (Fra-TXY per bacteria) were extracted from five bacteria as described (Akış et al., 1990). Briefly, Fra-T was obtained as supernatant by solubilization of bacteria in veronal buffer; Fra-X and Fra-Y were obtained by solubilization of the pellet at either at 95°C for or pH 2, respectively. Pooling of the three extracts were named as Fra-TXY. Addition to Fra-T, Fra-X, Fra-Y fractions for *S. typhimurium*, its outer membrane antigens were further fractionated as described (Barber et al., 1966). Protein-polysaccharide complexes were obtained from the supernatant of Fra-T following protein precipitation using trichloroaceticacid at 10% final concentration, and dialyzed against distillated water (Fra-A). The pellet was solved at natural pH and then divided for further extraction. One aliquot was precipitated and the supernatant was obtained (Fra-B) as described above. Another aliquot was precipitated using ammonium sulphate and proceeded as described above (Fra-C). Polysaccharide solution was obtained from Fra-A following hydrolysis of the fraction by boiling at 100°C for 90 min at final 5N acetic acid solution. The supernatant was dialyzed and kept (Fra-PS, polysaccharide fraction). Acid soluble flagella fraction was obtained by treatment of acetone-dried bacteria with HCl solution at pH 2 for 30 min. The supernatant was removed following ultra centrifugation at 100.000 g (Fra-D). The pellet was incubated with PBS and the supernatant was kept (Fra-ACF). A bacterial polysaccharide was purchased (Difco) and water solution was used in experiments (5 mg/ml).

SDS-PAGE

The samples were run on polyacrylamide gels in different concentrations varied between 7-14% in TRIS-buffer as described (Akış et al., 1990).

ELISA

Protein concentration of antigen solutions was determined by spectroscopy prior to the enzyme-linked-immuno-sorbent-assay (ELISA). Optimal antigen concentration and sera dilution were used in the tests. Microplates (Greiner, Frickenhausen, Germany) were coated with 100 ml antigen solution in PBS with overnight incubation in refrigerator, blocked with 2% BSA at 37°C for 2 hours, and stained with immunoglobulin in sera at 37°C for 1 h. After conjugation with anti-rabbit antibody (Institute Pasteur, Paris, France) and washing using 1% Tween 20-PBS, color development was measured at 20 min.

All assays were performed with multiple aliquots and confirmed. For ELISA a minimum of a two-fold difference was judged to be positive.

Table 1: Protein content of fractions.

Fraction	mg/g[a]	mg/g[b]
Fra-X		12
Fra-Y	4.1	
Fra-A		18.89
Fra-B	2.55	
Fra-C	8.36	
Fra-D	50.62	
Fra-ACF	29.96	
Fra-T		48
Fra-TXY (*S. typhi*)		42.4
Fra-TXY (*S. paratyphi-B*)		83.76
Fra-TXY (*E. coli*)		55.2
Fra-TXY (*S. flexineri*)		105.6

[a]Protein (mg)/1 g acetone-dried bacteria

[b]UV absorbing material (mg)/1 g acetone-dried bacteria

Results for protein content of each antigenic fraction were presented. The fraction names were enlisted at the left, and protein contets of the fractions in two different expressions were indicated at right colums.

Results

In electron microscopic examination of bacteria, the outer membrane has been found compact in 95% of the bacteria, and pillus and flagellar components were visible. During the immunization and at the time of blood drawn the animals appeared grossly normal. Animals developed high titer serum antibodies as detected by immunodiffusion (data not shown).

The protein yield of different bacteria and different fractions varied as shown in Table 1. In cross examination of immune sera against Fra-TXY from various bacteria and commercial polysaccharide, a strong cross reaction was detected against *S. paratyphi-B* antigenic fraction by immunodiffusion. Relatively weak cross reaction between *S. typhimurium* antigen and sera against *S. typhi*, and vice

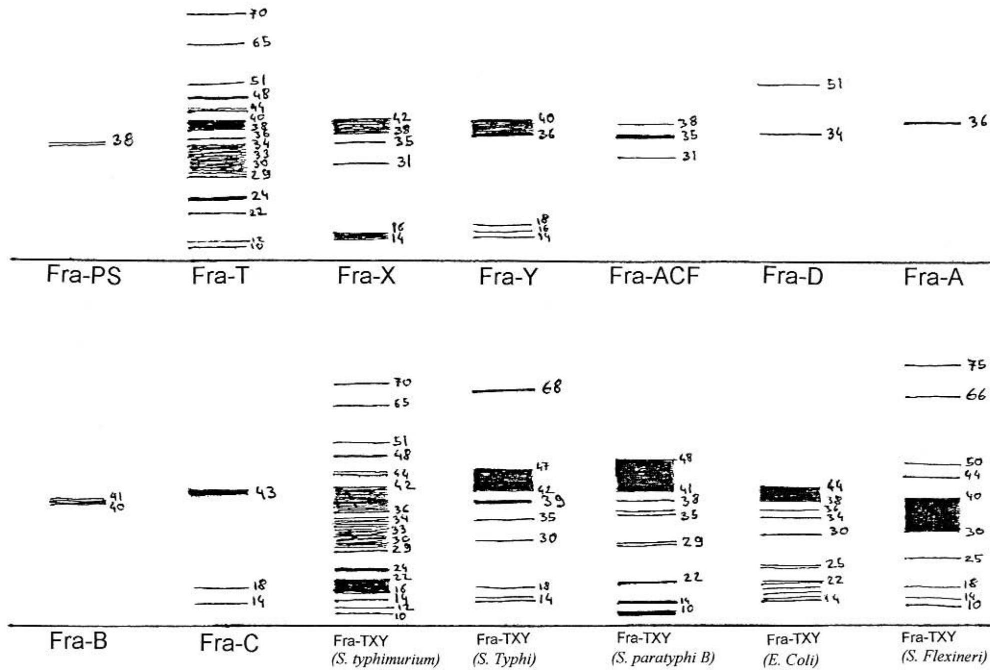

Figure 1: Protein band profiles in SDS-PAGE. The fractions were run in parallel and the band profiles were shown in picture. The sizes were indicated on profiles. The name of each fraction was indicated at the bottom of each profile.

versa, were observed (data not shown). The results were confirmed by ELISA. In further investigation by ELISA, cross reactions between commercial sera against *S. flexineri* and Fra-TXY antigen for *S. typhimurium* and *S. paratyphi-B* were observed.

14 antigenic fractions were evaluated by SDS-PAGE (Figure 1). Shifting of bands at 30 kDa to 31-42 kDa was observed when the denaturation condition was shifted to 100°C for 5 min from 60°C for 10 min. This was obvious particularly for Fra-C suggests this

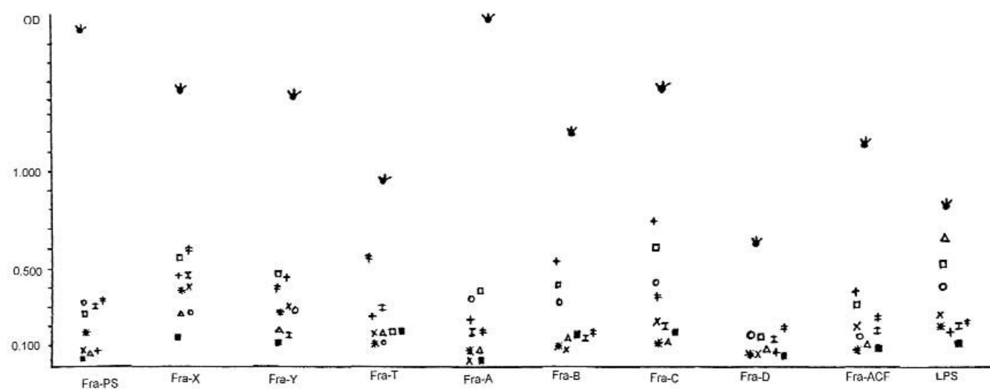

Figure 2: ELISA results obtained with *S. typhimurium* fractions. Each column presents OD values obtained with each fraction, and the name of each fraction was indicated at the bottom of each column. OD values were differentially dotted on diagram, and each dot indicates a different sera. See Figure 3 for description of the sera.

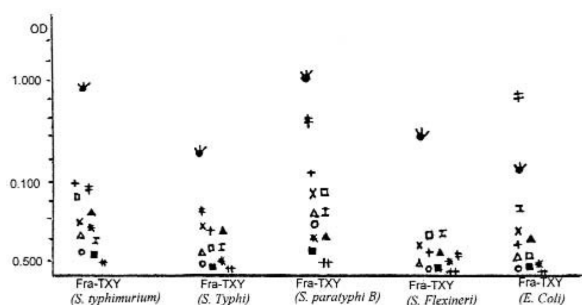

Figure 3: ELISA results obtained with Fra-TXY fractions for five bacteria. Each column presents OD values obtained with each fraction, and the name of each fraction was indicated at the bottom of each column. OD values were differentially dotted on diagram, and each dot indicates a different sera. Description of the sera was shown at the bottom of the diagram.

fraction contained outer membrane proteins, porins. A shift was also observed in Fra-X from 25-29 kDa to 33-35 kDa. Presence of polysaccharides in Fra-A was examined by hydrolysis. Band at 36 kDa shifted to 38-42 kDa after hydrolysis suggested protein was tightly bound to polysaccharide (Arockiasamy et al., 2000).

A 70 kDa *S. typhimurium* specific antigen was detected. 36-43 kDa proteins were common among five bacteria examined and could be responsible from cross reactions. 38 kDa band in Fra-PS; 36 kDa band in Fra-A; 34 and 51 kDa in Fra-D, 41-41 kDa in Fra-B, and 43 kDa in Fra-C were clear bands suggests they can be used in ELISA experiments. 51 kDa in Fra-D would be expected to be phase-1 pillus antigen (Prager et al., 2003).

ELISA test was optimized to obtain around 1.000 optic density (OD) for positive and around 0.100 OD for negative control. In this sensitivity level it was expected that high dilution antigen and sera would not allow non-specific cross reactions. In ELISA, polysaccharide fraction gave highest cross reaction with other bacterial sera suggests family specific epitopes dominate in this chemical structure (Figure 2).

Immune sera showed dramatically higher affinity to the *S. typhimurium* fractions than other sera did, suggests some antigenic epitopes were lost during preparation of O- and H-antigens (Figure 3). Conservation of original epitopes on acetone-dried bacteria was shown elsewhere (Akış et al., 1990). Fra-A showed highest antigenicity in ELISA whereas Fra-D the lowest one.

Discussion

There are many advantages of using immunochemical techniques in diagnostic microbiology laboratories, particularly in evaluation of post-vaccination sera in big size. The fractions described here should be particularly useful for examination of *Salmonella* vs. unrelated bacteria. Isolation of pure antigenic fractions has been more difficult than for polysaccharide fractions. We used multiple step isolation strategy to obtain pure fractions, and the methods should be reproducible by others. Although not all of the isolation methods were rigorously tested, the overall approach was relatively simple, straightforward and yielded unequivocal results. It will be briefly summarized.

Nine fractions were extracted successfully from *S. typhimurium*. In SDS-PAGE a 70 kDa specific antigen was detected in Fra-T, however it was not isolated in this study. 70 kDa protein seems a candidate antigen for using in immunochemical assays. Developing and using immunochemical techniques detecting diagnostic markers for gastroenteritidis, such as ELISA, have diagnostic value particularly in countries where gastroenteritis is endemic (Sood et al., 2002; Purwaningsih et al., 2002; Handojol et al., 2000). For this reason, description and isolation of species and serovar specific antigens is important. Many have been described and were successfully used by others (Veling et al., 2001; Solano et al., 2000; Arockiasamy et al., 2000).

S. typhimurium showed high cross reactivity to *S. paratyphi-B*, and relatively lower to *S. typhi*. 36-43 kDa antigens were common antigens to the bacteria tested, and could have diagnostic value in detection of family specific antigens in patient material. Fra-A showed pure band and highest score in ELISA suggested this fraction is a good candidate for this purpose. Others described cross reaction between *Salmonella* species (Valdirieso-Garcia et al., 2001), and used family specific antigens in ELISA tests (Jauho et al., 2000; Wang et al., 2000).

Strong immune response against acetone-dried bacteria, similar to natural infection, was shown previously using Fra-TXY as antigen in ELISA (Akış et al., 1990). In this study, fractions extracted from acetone-dried *S. typhimurium* under mild conditions were also found displaying strong antigenicity suggests antigens in these fractions conserved original epitopes during the extraction procedure.

Shift in bacterial wall protein bands in SDS-PAGE, particularly those among polysaccharide linked outer membrane proteins has been described (Sood et al., 2002). When the chemical bound in protein-polysaccharide complex was strong, hydrolysis of polysaccharide residues would not change the location of bands in SDS-PAGE. On the other hand, outer membrane proteins would easily shift. Both of the cases were evident in different fractions of this study. The protein content of a cell could also affect the chemical construction and rigidity of outer membrane. Among the bacteria, which were used in this study, dramatic difference was detected in protein content. This could be due to intrinsic properties of each bacterium.

Antibodies in homolog and heterolog O- and H-sera, which were prepared according to classical Grubel-Widal test, did fail to show high affinity to antigens, whereas in-house product hyperimmune sera reacted strongly. It is possible that some antigenic epitopes might be modified during the preparation of O- and H-antigens and might cause the weak reaction. On the other hand, *S. typhimurium* H-sera showed selective affinity to protein fractions as expected, however *S. paratyphi-B* O- and H-sera was not selective for protein or polysaccharide fractions. The value of classical Widal test has been discussed (Willke et al., 2002), and cross species polysaccharides in O-antigen were described (Wang et al., 2000). In these studies and others, Widal test was described as a supportive test rather than being an accurate test due to low sensitivity and specifity, however description of antigens according to Kaufmann-White schema has been continued for years (Popoff et al., 2001). As a results, new techniques have replaced the place of Widal test today. Nevertheless additional studies will be required to determine whether these sera are specific to pure protein or polysaccharide antigens.

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