Serological cross-reactions of *Proteus morganii* strains

Ph.D thesis

Zoltán Péterfi, M.D.

Head of the Doctoral School: János Szolcsányi, M.D. Academician
Program leader: Levente Emődy, M.D., PhD., D.Sc.
Tutor: Béla Kocsis, M.D., PhD., Med. Habil.

University of Pécs, Faculty of Medicine
Department of Medical Microbiology and Immunology
2007

1. Introduction

Identification and classification of somatic O antigens is one of the most commonly used method for typing of Gram-negative bacteria. It is based on the serological and immunological detection of differences in the antigenic composition of the cell envelope. The immunodominant molecule is the lipopolysaccharide (LPS), specifically the O-side chain. It is made up of oligosaccharide repeating units and their structures vary according to the different serogroups. The other main component of the outer membrane, namely the outer membrane protein composition, also allows differentiation between Gram-negative species.

In 1967 Rauss, Ralovich and Vörös described antigenic relationship between *Proteus (Morganella) morganii* O34 (8662/64) and *Escherichia coli* O111 based on tube agglutination. However, no detailed information about the lipopolysaccharide composition was given. Another cross-reaction was described earlier between *E. coli* O111 and *Salmonella* Adelaide O35. These two bacteria have important pathogenicity. The *E. coli* O111:K58:B4 serotype was among the first *E. coli* strains identified as a cause of infantile diarrhea and it is the second most frequently isolated seroptype causing outbreaks of haemorrhagic colitis with life threatening haemolytic uremic syndrome (HUS).

More profound understanding of these cross-reactions may improve the interpretation of serodiagnostic tests. Immunochemical studies of LPS are important for substantiation of serological cross-reactivity between representatives of different taxa at the molecular level. The cross-reactive antibodies recognize identical or similar disaccharide to a tetrasaccharide fragment in the oligosaccharide repeating units of LPS. While serological cross-reactivity between common pathogens might cause difficulties in differential diagnostics of infections. This phenomenon can have diagnostic value if one of the cross-reacting species is not pathogenic and is unlikely to enter the host. Under these conditions, cross-reacting antigens obtained from the non-pathogenic strain can be used for the diagnosis of infections caused by the pathogenic counterpart. Study of cross-reacting antigens may be considerably beneficial in the search for candidate vaccine strains against pathogenic bacteria.

O antigenic cross reactions between Gram-negative species due to LPS chemical composition have been repeatedly demonstrated over the years. There are only three cases in which the O-antigenic structures have been shown to be not only closely related but identical: (1) *E. coli* O111 and *S. enterica* O35, (2) *E. coli* O55 and *S. enterica* O50 and (3) *E. coli* O157 and *S. enterica* O30.

2. Aims of this study

Our aims were:

1. methodological improvement of ELISA for LPS, eligible for detection of minor differences between antigens
2. to test the clinical applicability of our new ELISA method
3. purification and identification of the cross-reacting antigens
4. to determine the serological characteristics of *Proteus morganii* O34 (8662/64) LPS and to compare it with cross-reactive lipopolysaccharides from *E. coli* O111 and *Salmonella* Adelaide O35
5. structural analysis and comparison of heat stabile, lipopolysaccharide antigens
6. homology analysis of the *rfb* gene clusters of the studied strains
7. identification of cross-reacting heat labile, protein antigens
8. to analyse possibilities for vaccination
9. to expand the scope of our experiments to further bacterial genera in order to uncover additional serological relationships

3. Materials and methods

3.1. Bacteria and antigens

Proteus morganii O34 (8662/64) O1, O9 and O43, *Escherichia coli* O21, O22, O33, O61, O68 and O111, *Salmonella* Urbana O30, *Salmonella* Adelaide O35, *Yersinia enterocolitica* O9, *Shigella sonnei*: phase I., phase II. and Re 4350, *Bacteroides fragilis* strains were used.

Bacteria were grown in a fermentor at 37°C (Braun Melsungen-Biostat U 30, Germany). The **lipopolysaccharides** (LPS) were prepared by **phenol-water method** from S-form bacteria**, and** by **phenol-chlorophorm-petrolether** method from R-form bacteria.

Preparation of outer membrane proteins: Bacteria were cultivated in 2000 ml of tryptic soy broth (TSB) with shaking (100 rpm) at 37°C for 24 h, collected by centrifugation at 5000 g for 15 min at 4°C, and washed twice with physiological saline. The outer membrane proteins (OMP) were extracted by Osborn's method.

3.2. Electrophoresis of antigens

SDS-PAGE was carried out in a Laemmli discontinuous system in a 12% separation gel. The gels containing **protein samples** were stained by Coomassie Brilliant Blue R-250. The gels containing **LPS samples** were transferred to a fixing solution overnight before silver staining by the method of Hancock and Poxton.

3.3. Production of antisera

Bacteria used for immunization were grown on agar medium at 37 °C overnight, washed and adjusted in saline to give 1×10^8 cfu/ml. This suspension was boiled at 100 °C for 1 h. New Zealand rabbits (mean weight 3 kg) were immunized intravenously with the above bacterial suspension.

Rabbit antibodies were raised also against **outer membrane proteins**. The extracted OMPs were separated by SDS-PAGE, blotted to nitrocellulose. The nitrocellulose strips containing the proper antigens were dried, dissolved in DMSO and mixed 1:1 with complete Freund's adjuvant. One milliliter of this emulsion was injected subcutaneously into the interscapular region, 1 ml intraperitoneally and 0.5 ml intramuscularly into each rear thigh. Booster injection was given 2 weeks later, then monthly, for 4 months.

3.4. Tube agglutination

Doubling dilutions of antisera in 1 ml physiological saline were tested starting with a dilution of 1 in 100. 25 µl of the bacterial suspension (A_{600}=1.2) was added to each tube including a control one containing only saline. Agglutination was read by an agglutinoscope after incubation at 37°C for 24 h, and the endpoint titer was defined as the highest dilution of antiserum where agglutination could be detected.

3.5. Passive haemagglutination:

The haemagglutination experiments were performed in Takátsy microplates. Sheep erythrocytes were sensitized with LPS (1.5 mg/ml) in a 37 °C-water bath for 1 h. Red blood cells were washed three times with physiological saline and used as a 5% suspension added to doubling and triplicate serial dilution of sera. Agglutination was recorded after incubation at room temperature for 10 h.

3.6. Direct ELISA

The technique was originally described by Engvall and Perlmann, and modified by Takahashi. To achieve a better applicability for LPS analysis we further modified the method.

Precoating of microplates: A solution (100 μl) of poly-L-lysine (MW.: 260 000) (Sigma Chemicals, St. Louis, MO, USA) (10 μg/ml) in 0.01 M phosphate-buffered saline at pH 7.2 (PBS) was placed in polystyrene microplates (Nunc Immunoplate, Intermed, Denmark). The solution was incubated overnight at room temperature.

Binding of LPS: Aliquots of various concentrations of LPS (100 μl) suspended in PBS were placed in poly-L-lysine precoated plates and then incubated for 1 h at 37 ºC. The plates were washed four times with PBS containing 0.05% Tween 20 (T-PBS).

Our methodological modification for blocking the non-specific binding sites: Casein, BSA, goat, sheep, pig, bovine, guinea pig, horse sera were used for blocking non-specific binding sites. Aliquots of different dilutions of 0.5% casein or BSA or sera (200 μl) were placed in each well, incubated for 30 min at 37 ºC, and washed four times with T-PBS.

Antisera: A previously pre-titrated optimum dilution of rabbit immune serum (100 μl) was added to each well, incubated for 1 h at 37 ºC, and washed four times with T-PBS.

Conjugate: Aliquots (100 μl) of a 1/500 dilution of peroxidase conjugated anti-rabbit Ig G (goat sera) (Sigma Chemicals, St. Louis, MO, USA) were added to each well, incubated for 1 h at 37 ºC, and washed four times with T-PBS.

Substrate: The peroxidase substrate solution containing 0.01% o-phenylene diamine (Sigma Chemicals, St. Louis, MO, USA) and 0.03% H_2O_2 (100 μl) was added to each well. Reactions were stopped about 10 min later by 4 N H_2SO_4. The optical density (OD) was read at 492 nm in a Titertek Uniscan reader (Flow Laboratories, Helsinki, Finland).

Quality control: The photometer was blanked on air and the wells were read. The OD of buffer control was accepted only if it ranged between 0.000 and 0.099. If the value was above 0.100, inadequate washing was considered and the experiments were repeated. The difference between the OD value of the positive control and the negative control (IgG off) must have been at least 0.800. Only after these quality control criteria had been fulfilled, the photometer was blanked on the negative control, and the experiment was evaluated.

Investigations with patients' sera: When antibody titres in human sera were investigated peroxidase conjugated anti-human immunoglobulin M (IgM) and mixed peroxidase conjugated anti-human immunoglobulins IgG, IgM (Sigma Chemicals, St. Louis, MO, USA) were used for detection of anti-lipopolysaccharide immunoglobulins from examined patients. The cut-off level for a positive reaction was determined at 0.3 optical density previously calculated as the level three times above the standard deviation of negative controls. Criterion for an enhanced titer was an optical density at least twice above the standard deviation of negative controls.

3.7. Immunoblot:

Electrophoretic transfer of LPS and proteins from SDS-PAGE gel to nitrocellulose sheets of a 0.2 μm pore size (Sigma Chemicals, St. Louis, MO, USA) was performed at 350 mA in electro-blotting apparatus (Bio-Rad Laboratories, Richmond, CA, USA) for 60 min at 4°C as described by the supplier.

3.8. Thin layer chromatography (TLC)

TLC plates were running in a Shandon's chromatography vessel. Monosaccharide sample solution of hydrolyzed LPS was chromatografied in Cellulose DC Plastikfolia (Merck, Darmstadt, Germany) thin layer with running stage: n-butanol-pyridine-water (6:4:3) for neutral-, and pyridine-ethyl acetate-water-acetic acid (5:5:3:1) for aminosugars. A mixture of D-ribose, D-mannose, D-galactose, D-glucose, D-heptose, D-glucosamine and D-galactosamine (Supelco, Bellefonte, PA, USA) was used as standard. The samples were developed by silver staining described by W. E. Trevelyan et al.

3.9. Gas chromatography-mass spectrometry (GC-MS)
The sugar composition of LPS was analyzed after hydrolysis of polysaccharide in alditol acetate form of monosaccharides. We used an Agilent GC-MS (Agilent technologies, USA) gas chromatograph-mass spectrometer system equipped with a 30 m long, internal diameter 251 µm capillary column Agilent DB-225 HP-1MS (Agilent Technologies, USA), containing OV-225 liquid phase. 15 ml/min helium was used as a carrier gas. The temperature was 220 °C in the injector and 180 °C in the column. As an internal standard we used mesoinozit.

3.10. Column chromatography
Degradation products of LPS such as polysaccharide (DPS) and Lipid-A were prepared by mild acid hydrolysis with 1% acetic acid (100 °C, 90 min). The precipitate was removed by centrifugation. The supernatant was fractionated by GPC on column (2.5x80 cm) of Sephadex G-50 (Sigma Chemicals, St. Louis, MO, USA) using pyridine-acetic acid buffer to prepare purified O-specific polysaccharide and core oligosaccharide.

3.11. NMR
^{13}C-NMR spectra were recorded with Varian ^{UNITY}INOVA 400 WB (100 MHz for ^{13}C) spectrometer. Measurements were run at 303K probe temperature and were made at concentrations of 8 mg/ml in D_2O. Acetone was used as internal reference (δ_C = 31.07 ppm). During the experiment 46,000 free induction decay signals (FID's) were acquired. Measurements were run with 64,000 datum points using a 30° (2.5µs) pulse and a relaxation delay of 3s. Prior to Fourier transformation, the FID's were multiplied exponentially (using 3Hz line broadening) and were twice zero-filled.

3.12. Capillary electrophoresis
Capillary electrophoretic measurements were performed with the Biofocus 3000 system (BioRad Laboratories, Hercules, CA, USA) applying "dynamic sieving" CE. Molecular weight of the separated proteins was evaluated by means of the low molecular weight calibration kit of Pharmacia. All experiments were repeated three times.

3.13. Lab-on-a-Chip technology
Microchip-based measurements were performed in the commercially available Agilent 2100 Bioanalyzer system, applying the Protein 200 Plus LabChip Kits. Chips were treated according to the protocol provided with the Protein 200 LabChip Kit.

3.14. Growth inhibition
Parallel series of serum dilutions were prepared in nutrient broth medium (1 ml) in test tubes. To each tube 10 µl of standardized complement (Institute Virion Ltd., Switzerland) and 5 µl of bacterial suspension (A_{600} = 0.6) were added. Absorption was read after incubation at 37 °C for 24 h.

3.15. Opsonic function of antibodies
10 µl of each bacterial culture A_{600} = 0.5 was added to monolayers of RAW264.7 macrophage cells. For opsonisation bacterial suspensions were mixed with a serum sample of 1:1000 dilution (complement inactivation by 56°C, 30 min.). After a 30 min uptake, a gentamicin solution was added to the medium (final concentration 80 µg/ml) to kill the extracellular bacteria. Internalized bacteria were enumerated as colony-forming units by plating of detergent-lysed (1% Triton X-100 - Sigma Chemicals, St. Louis, MO, USA) macrophages onto nutrient agar.

3.16. Genetic examination
The gene cluster in O antigen of *E. coli* O111 and *S.* Adelaide O35 is well known and accessible in the GeneBank (AF078736, AF285969). Using primers corresponding to *man*B gene of *E. coli* we performed PCR amplification and sequential analysis.

3.17. Statistical analyses were performed using the Origin 7.5 graphical and statistical program (OriginLab Corporation, Northampton MO, USA).

4. Results

4.1. Methodological improvement of ELISA for LPS, eligible for detection of minor differences between studied antigens

Optimization of LPS concentrations: Microplates were coated with various concentrations of *S. sonnei* phase I LPS. Proper extinction values for evaluation were obtained with LPS concentrations higher than 0.1 μg/ml. For further experiments we used LPS concentration at 1 μg/ml.

Optimization of blocking: Microplates were coated with *S. sonnei* phase I. LPS at 1 μ g/ml. Non-specific binding sites were saturated with varying dilutions of casein (0.5%), BSA (0.5%) or sera of different animal species. Minimum OD background was obtained with a serum dilution at 1/20 and a casein and BSA dilution at 1/100 respectively. We used these dilutions for further experiments.

Comparison of various blocking agents in ELISA: Casein, BSA, goat, sheep, pig, bovine, guinea pig or horse sera were tested. The traditional casein and BSA were compared to animal sera. When using goat serum as blocking agent significantly lower OD values were measured for the negative control ($p<0.001$) and significantly higher OD values for the positive control ($p<0.001$). No statistical differences could be fined when comparing the blocking effect of sera originating from different animal species.

Precision data: Intra-assay variations were determined using 6 simultaneous determinations (SD 0.033 for positive controls, and SD 0.023 for negative controls) using goat serum in comparison to casein or BSA. Inter-assay variations were determined by testing 12 determinations performed individually (SD 0.088 for positive controls, and SD 0.097 for negative controls) using goat serum in comparison to casein or BSA.

Reproducibility of data: It is well known that there is an individual variation in protein, immunoglobulin concentration of sera. For excluding the interference of this factor with the confidence of our results we compared goat sera from 20 different animals. No significant variation was obtained (SD 0.044 for negative, and 0.080 for positive control).

Competitive re-blocking test: In simultaneous tests non-specific binding sites were blocked, firstly with casein, BSA or goat serum. In the next step, wells blocked with casein or BSA were re-blocked with goat serum. Alternatively, wells blocked with goat serum were re-blocked with casein or BSA. When goat serum was used for re-blocking, the background values were significantly lower ($p<0.05$) than in the case of a simple blocking with either casein or BSA. If casein or BSA were used for re-blocking, the background extinctions were significantly higher ($p<0.05$) than for goat serum as the only agent.

Comparison of various LPS forms: LPS extracted from different smooth and rough strains such as *S. sonnei* phase I and II, Re 4350, *Escherichia coli* O21 and O111, *Salmonella* Urbana O30, *S.* Adelaide O35, *Yersinia enterocolitica* O9, and *Proteus morganii* O1, O9 and O43 were used for testing the applicability of our modified ELISA method. Goat serum

proved to be the best choice for blocking also in these assays for both smooth and rough strains.

Cross reactivity analysis of LPS: In our experiments antisera to *S. sonnei* phase I cross reacted with *S. sonnei* phase II LPS, but not with *S. sonnei* Re 4350 LPS. Antisera against *S. sonnei* phase II and *S. sonnei* Re 4350 cross reacted with *S. sonnei* phase I, *S. sonnei* phase II, *S. sonnei* Re 4350 LPS, too, showing that these antigens contain common structural components and are capable to elicit serological cross reaction. The superiority of goat serum as blocking agent could be shown also in these experimental settings established for studying antigenic relationships between representatives of various bacterial genera.

4.2. Clinical applicability of our modified ELISA method

Using our modified ELISA test we investigated the possible relations between the histological image of the appendix and seroconversion of patients with appenidicitis to representatives of their intestinal microbiota. The first serum was collected on day of operation, the second one 14 days later. Parallel with severity of the inflammation we could detect significantly higher antibody levels in the patients when compared to the controls ($p<0.001$ against *E. coli,* $p<0.005$ against *B. fragilis*).

We could show enhanced antibody titres against the LPS of the anaerobic *Bacteroides fragilis* in most patients with simple and phlegmonous appendicitis. On the other hand for *E. coli* we observed increasing antibody levels parallel to the severity of the inflammation. The increasing antibody titres correlated with the time elapsed from the onset of the disease.

The high number of patients with positive serologic test against absolute rough mutant LPS of *Shigella sonnei* Re 4350 (16 from 24 cases) indicates a frequent response to this less specific but more common LPS component of Gram-negative bacteria.

4.3. Purification and identification of cross-reactive antigens

Preparation of lipopolisaccharides and outer membrane proteins

Extracted and purified lipopolysaccharides were used for further examination in undigested form or after partial or complete hydrolysis. Hydrolyzed compounds were purified with column chromatography method. Preparation of bacterial outer membrane proteins (OMP) was carried out as described in Materials and Methods.

4.4. Serological characteristics of *Proteus morganii* O34 (8662/64) LPS and comparison with cross-reactive lipopolysaccharides extracted from *E. coli* O111 and *Salmonella* Adelaide O35

Confirmation of serological cross-reaction between the three strains:

Using bacterial **tube agglutination** we were able to reproduce the results of cross-agglutination test carried out by Rauss, Ralovich and Vörös. For proper serological analysis of LPS we used purified LPS extracted from these bacteria. The results of **passive haemagglutination** showed several serological differences between strains, but considerable cross-reaction as well. The antibodies produced to *P. morganii* O34 showed high reactivity with *S.* Adelaide O35 LPS. No reaction occurred in a reciprocal setting.

ELISA microplates were conjugated successively with LPS, DPS and Lipid-A extracted from *P. morganii* O34*, E. coli* O111 and *S.* Adelaide O35 strains and then incubated with sera produced against these bacteria. The intensity of reactions with *E. coli* O111 and *S.* Adelaide O35 LPS seemed similar (OD 1.149-1.361), but with *P. morganii* O34 LPS was slightly lower (OD 0.87-1.09) what was perhaps due to its lower DPS content. We could not find differences in reactions between *P. morganii* O34 LPS and different studied sera. *E. coli* O111 and *S.* Adelaide O35 LPS also gave similar reaction with any cross-reacting sera. Sera

cross-absorbed with whole heat-killed cells (100°C, 60 min.) of these strains was also used. For detection of minor antigenic differences and characteristics we used serum dilution too. No differences between reactions were observed. So we could detect serological cross-reaction between the three strains by ELISA test using LPS-s and their components.

4.5. Chemical background:

LPS extracts of all strains gave characteristic silver-stained ladder pattern in **SDS-PAGE**. The profiles of *P. morganii* O34, *E. coli* O111, *S.* Adelaide O35 LPS were similar in terms of relative spacing and distribution of bands, as it would be expected if they shared the similar O antigen structure and the three profiles could be superimposed. Serological and structural similarity between these strains was proved by **immunoblotting** too. LPS ladders of the three strains were revealed also by using *P. morganii* O34, *E. coli* O111, *S.* Adelaide O35 antiserum. The results support the conclusion that the serological cross-reacting structure of these strains is the O antigen. Furthermore, immunoblotting with absorbed antisera provided additional evidence of the involvement of O antigen determinants.

In the next step monosaccharides obtained with acid hydrolysis were separated **by thin layer chromatography**. We found two spots of neutral sugars at the line of galactose and glucose standards. Mannose is absent from each lipopolysaccharide. We couldn't visualize colitose (3,6 dideoxy-L-galactose) because the loss of their -OH radicals confers the ability to run more quickly and superpose with glucose. After calculating the area and intensity of glucose and galactose spots we obtained the proportion of glucose (containing colitose) and galactose about 3 to 1, namely the proportion of these sugars known in repeating units. In the absence of sufficient amount of colitose as standard, we could not detect exactly this rare sugar on TLC plate.

Gas chromatographic spectra for the studied LPS preparations showed similarity of their monosaccharide contents. Mass spectrometric analysis of colitose (received as a generous gift from prof. Stefan Oscarson (Stocholm University, Inst. Org. Chem, Sweden) was eligible for identification of this rare sugar in our three LPS.

With **^{13}C-NMR** spectrometer we were able to detect colitose in all the three lipopolysaccharides studied. The spectrum of DPS polysaccharide prepared from *P. morganii* O34 LPS is similar to spectra from *Escherichia coli* O111 and *Salmonella* Adelaide O35, however, the determination of fine structure of these LPS-s needs further ^{13}C-NMR analysis.

4.6. Genetic background

Preliminary studies in sequence analysis of the *manB* gene, a representative from the O gene cluster showed 70% similarity between *P.* morganii O34 (8662/64) and *S.* Adelaide O35, and 69% similarity between *P. morganii* O34 (8662/64) and *E. coli* O111, respectively. We made the *P. morganii* O34 (8662/64) *manB* gene sequence available in GeneBank with accession number EF051576.

4.7. Identification of cross-reacting protein antigens

Capillary electrophoretic OMP profiles

Capillary electrophoretic OMP profiles of the three strains were dominated by the region from 35 to 50 kDa, and showed close correlation with the microchip based electropherograms. Profile of *E. coli* O111 consisted of more characteristic peaks with three dominating proteins of 36, 40, and 45 kDa molecular masses. Pattern of *P. morganii* O34 (8662/64) revealed two characteristic proteins of 36 and 41 kDa. The OMP pattern of *S.* Adelaide O35 contained more major protein peaks, but the presence of the common dominating proteins and the major 39 kDa protein could be detected in this pattern as well. Quantitative evaluation of protein peaks

(peak height and peak areas) was rendered more difficult by the baseline drift observed in capillary electrophoretic patterns.

Chip-based electropherograms of the outer membrane proteins

Microchip-based patterns of all three strains contained the dominating protein peaks in the 35-50 kDa molecular weight range. In the profile of *E. coli* O111 three protein peaks with molecular weights of 36, 41, and 45 kDa could be distinquished, the quantity of the 36 kDa protein was much higher, than that of the others. In the same region of *P. morganii* O34 (8662/64) two major OMPs with apparent molecular weights of 36 and 41 kDa, respectively, could be detected. The pattern of *S.* Adelaide O35 was dominated by a protein with molecular weight of 39 kD being not present in the patterns of the two other strains. However, the three proteins with molecular masses of 36, 41 and 45 kDa could also be detected in the OMP profile of *S.* Adelaide O35.

The presence of common protein antigens (36 and 41 kDa) in the OMP patterns of the three serologically cross-reacting strains was demonstrated by the simultaneous injection of the samples: the peak level of common proteins increased.

SDS-PAGE and Western blot analysis

One-dimensional SDS-PAGE of outer membrane proteins extracted from the analyzed strains was performed. The dominating regions containing the major OMPs could be identified by this technique as well. The 36 and 41 kDa protein bands were cut out and used to produce polyclonal in rabbits to examine the antigenic relationships. After blotting of the electrophoretically separated outer membrane proteins on nitrocellulose membrane, the sera produced against the OMP components of *P. morganii* O34 (8662/64), *E. coli* O111 and *S.* Adelaide O35 strains were applied consecutively against all three strains. The immune sera showed high cross-reactivities for all three strains indicating the antigenic relatedness of their outer membrane proteins with identical molecular masses.

4.8. Possible applicability of the cross-reactive antigens in vaccine development

Growth inhibition studies: While no significant inhibition of growth occured for the three investigated strains in the presence of complement and normal rabbit or human sera, a significant restriction of growth occured ($p < 0.001$) when cross-reactive sera and complement were added to the cultures of any of the three bacteria.

In an uptake assay bacterial uptake by RAW264.7 macrophages significantly increased ($p < 0.001$) in the presence of specific or cross-reacting antisera as compared to the rate of uptake in the presence of non-specific sera.

4.9. New possibilities for exploration of other serological cross-reactions

Preliminary results of our ongoing experiments point to further antigenic relationships between ***P. morganii* O43:12 (Louvain) - *Y. enterocolitica* O9 - *E. coli* O157:H7 - *S.* Urbana O30 - *Brucella abortus*** just as between ***P. morganii* O9 (352/56) - *E. coli* O21;K20 - *S.* Inverness O38 - *Salmonella* O44 - *Citrobacter freundi* O14** strains. A third antigenically related group involves ***P. morganii* O29 (1594) - *E. coli* O112ac;K68 - *S. dysenteriae* 2.** As severe pathogens occur among these cross-racting bacteria not only a thorough analysis of their antigenic relations but also the possibility of a cross-protectivity is planned to be investigated.

5. Discussion and conclusions

1.-2. ELISA is a sensitive, specific, reproducible and fairly fast method. In case of LPS ELISA, problems exist, such as non-specific binding of antibodies to the plastic wells. For this reason we aimed to improve the applicability of this method for LPS analysis. Utilizing goat sera as blocking agent we could significantly decrease the background values in comparison with the originally and generally used casein or BSA. This new blocking technique was eligible for detection of minor differences between LPS antigens as shown in the case of *S. sonnei* mutants or cross-reacting bacterial strains as well as in clinical samples.

3. We were able to reproduce serological cross-reactions described earlier by bacterial tube agglutination between strains of various enterobacterial genera. Meticulous examination of these cross-reaction was performed with more sensitive tests (e.g. indirect haemagglutinatoin, ELISA, immunoblot).

4.-5. Chemical examination (TLC) of LPS O side chains detected the presence of similar monosaccharide. Additionally the GC-MS and NMR spectra of the studied lipopolysaccharides were similar, and demonstrated that *P. morganii* O34 (8662/64) O side chain is made up from the same sugars, and with a similar structure as known in *E. coli* O111 and *S.* Adelaide O35 LPS: namely two colitoses (3,6-dideoxy-L-galactose) linked to glucose, galactose and N acetyl-galactosamine. Our study is the first report on the identitiy of the monosaccharide composition of the above three bacteria.

6. Gene sequencing of the *manB* gene from the O side chain gene cluster shows high sequence conservation for the studied bacterial strains.

7. The outer membrane protein components of the three strains were analyzed to prove their significant role in the serological cross reactions. Expression of a 36 and a 41 kDa protein could be detected in all the three electropherograms, and Western blot analysis revealed also an antigenic relationship between the corresponding proteins.

8. The results of in vitro bacterial growth inhibitory and macrophage uptake assays support the assumption that cross-reactivity might serve as a basis of preventive antibacterial methods.

9. The experiences of this study encourage us to continue our efforts to uncover further antigenic relationships for both basic analysis and possible practical applications.

List of publications and oral presentations

Papers serve as basis of this thesis:

1. **Péterfi Z**, Kocsis B: Comparison of blocking agents for an ELISA for LPS, J. Immunoassay 2000, 21(4): 341-354 **IF: 1,286**
2. **Péterfi Z**, Kocsis B: Hogyan válasszunk blokkoló anyagot az endotoxin-ELISA érzékenységének növeléséhez? Orvostudományi Értesítő 2000, 73:166-171
3. **Péterfi Z**, Kustos I, Kocsis B: Szerologiai keresztkapcsolatok vizsgálata a P*roteus morganii* - *Escherichia coli* – *Salmonella* Adelaide törzsek között. Orvostudományi Értesítő 2003/2; 76: 142-150
4. **Péterfi Z,** Kovács K, Antal A, Kocsis B: Anti-LPS antibodies in acute appendicitis dectected by ELISA. APMIS 2006; 114(4):265-9. **IF: 2,127**
5. **Péterfi Z,** Kustos I, Kilár F, Kocsis B: Microfluid chip analysis of outer membrane proteins responsible for serological cross reaction between three Gram-negative bacteria: *Proteus morganii* O34, *Escherichia coli* O111 and *Salmonella* Adelaide O35 J. Chromatography A, (accepted for publication) **IF: 3,096**
6. **Péterfi Z,** Ősz E, Reuter G, Kilár F, Kocsis B: Structural and serologic properties of O-specific polysaccharide from *Proteus morganii* O34 (8662/64) possessing cross-reactivity with *Escherichia coli* O111 and *Salmonella* Adelaide O35. J Chromatography B, under review (expected **IF: 2,391)**

Other important papers:

1. Nemes Zs**,** Kiss G, Madarassi PE, **Péterfi Z,** Ferenczi E., Bakonyi T, Ternák G**:** Nosocomial transmission of Dengue. Emerging Infect Dis. 2004; 10(10):1880-1 **IF: 5,643**
2. Nemes Zs, **Péterfi Z**: A *Campylobacter jejuni* enteritis, mint a dysenteria syndroma képviselője, Medicus Universalis XXXI/1, 1998, 13-15
3. Nemes Zs, **Péterfi Z**: Kullancs által közvetített újabb betegség - a humán ehrlichiózis, Praxis 1998; 7(11):56-58
4. Nemes Zs, **Péterfi Z**: Együttes leptospira- és hantavírus-fertőzés ugyanabban a betegben, Orv. Hetil 2000, 141 (10): 499-502
5. **Péterfi Z**, Nemes Zs: Leptospirosis és hantavírus fertőzés. Orvostudományi Értesítő 2000, 73:172-175
6. **Péterfi Z:** Emerging fertőző betegségek Orvostudományi Értesítő 2004/1; 77(1):52-62
7. **Péterfi Z:** Legionellosis és más atípusos pneumoniák, Magyar Orvos 2006; 15(5):18-25

   …

Abstracts:

1. **Péterfi Z,** Mazák I, Kocsis B, Vörös S: Immunochemical and serological analysis of *Proteus morganii* strains, Acta Microbiol. Hung. 1999. 46: 131
2. **Péterfi Z,** Kocsis B: Optimization of ELISA test used for detection of serological cross-reaction between lipopolysaccharides, Acta Microbiol. Hung. 2000. 47:215
3. Kocsis B, Kustos I, **Péterfi Z:** Isolation and characterization of a *Shigella sonnei* absolute rough mutant, Acta Microbiol. Hung. 2000. 47:217
4. **Péterfi Z**, Kovács K, Antal A, Kocsis B: Serological examinations in acute appendicitis Acta Microbiol. Hung. 2004. 51:156
5. **Péterfi Z**, Kustos I, Kocsis B: Serological cross reaction examination between P*roteus morganii* - *Escherichia coli* – *Salmonella* Adelaide Acta Microbiol. Hung. 2003. 50:231
6. Kilár F; Bui A; **Péterfi Z;** Kustos I; Kocsis B.: Bacterial Endotoxins. A complex study by capillary electrophoresis with laser induced fluorescence, Rome, Italy, ITP, 2004. - L 25

Oral and poster presentations:

1. **Péterfi Z**, Mazák I, Kocsis B, Vörös S: A *Proteus morganii* törzsek immunkémiai és szerológiai vizsgálata. oral presentation, Magyar Mikrobiológiai Társaság Nagygyülése, Miskolc, 1998. aug.
2. **Péterfi Z**, Kocsis B: Optimization of ELISA test used for detection of serological cross-reaction between lipopolysaccharides. poster, 13th International Congress of the Hungarian Society for Microbiology. Budapest. 1999. aug. 30

3. Kocsis B, Kustos I, **Péterfi Z**: Isolation and characterization of a *Shigella sonnei* absolute rough mutant. oral presentation, 13th International Congress of the Hungarian Society for Microbiology. Budapest. 1999. aug. 30
4. **Péterfi Z**, Kocsis B: Hogyan válasszunk blokkoló anyagot az endotoxin-ELISA érzékenységének növeléséhez? oral presentation EME Orvostudományi és Gyógyszerészeti Szakosztály X. Tudományos Ülésszaka, Székelyudvarhely, Románia 2000. Május 11-13
5. **Péterfi Z**, Nemes Zs: Leptospirosis és hantavírus fertőzés. oral presentation EME Orvostudományi és Gyógyszerészeti Szakosztály X. Tudományos Ülésszaka, Székelyudvarhely, Románia 2000. Május 11-13.
6. Nemes Zs, **Péterfi Z**, Ferenczi E: Zoonozisok együttes előfordulása betegeinkben poster Magyar Infektológiai Társaság éves kongresszusa, Budapest 2000. Okt. 12-14
7. **Péterfi Z**, Kustos I, Kocsis B: Szerologiai keresztkapcsolatok vizsgálata a P*roteus morganii - Escherichia coli – Salmonella* Adelaide törzsek között, poster Magyar Mikrobiológiai Társaság Nagygyülése, Balatonfüred, 2001
8. **Péterfi Z**, Kovács K, Antal A, Kocsis B: Acut appendicitis szerológiai vizsgálata oral presentation Magyar Mikrobiológiai Társaság Nagygyülése, Balatonfüred, 2002
9. **Péterfi Z**, Kustos I, Kocsis B: Szerologiai keresztkapcsolatok vizsgálata a P*roteus morganii - Escherichia coli – Salmonella* Adelaide törzsek között, oral presentation EME Orvostudományi és Gyógyszerészeti Szakosztály XIII. Tudományos Ülésszaka, Sepsiszentgyörgy, Románia, 2003. Május 29-31
10. Bui A, **Péterfi Z**, Kustos I, Kocsis B, Kilár F: Analyzation of 8-aminopyrene-1,3,6-trisulfonic acid-labelled hydrolized endotoxins by capillary electrophoresis, poster 5th International symposium and course, Teaching and learning of „analytical and bioanalytical monitoring methods" 2004 31 May-07 June Sofia, Bulgaria
11. Bui A, **Péterfi Z**, Kustos I, Kocsis B, Kilár F: Optimization of experimental conditions in the analysis of 8-aminopyrene-1,3,6-trisulfonic acid-labelled hydrolyzed endotoxins by capillary electrophoresis poster 14th International Symposium on Capillary Electroseparation Techniques, Rome, Italy, September 12-15, 2004
12. Kilár F, Bui A, **Péterfi Z,** Kustos I, Kocsis B: Bacterial endotoxins. A complex study by capillary electrophoresis with laser induced fluorescence oral presentation 14th International Symposium on Capillary Electroseparation Techniques, Rome, Italy, September 12-15, 2004

Publications

Book chapters (Betegség enciklopédia, Springer kiadó, 2002): **19**
Total papers: **25**
Oral and poster presentations: **42**
Cumulative impact factor: **13,016 (+2,391** under review**)**
Independent citations: **20**