Lipopolysaccharide microarrays for the detection of antibodies

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Abstract

Lipopolysaccharide (LPS) is the major component of Gram-negative bacterial outer membrane. LPS are immunogenic and show species/strain specificity. The demonstration of anti-LPS antibodies in clinical samples is of diagnostic value in certain Gram-negative bacterial infections. In the present study we explored the possibility of immobilizing LPS isolated from different bacteria in a microarray format for the detection of anti-LPS antibodies. LPS was successfully immobilized on nitrocellulose-coated glass slides, preserving the accessibility of epitopes for antibody binding. Specificity of the LPS arrays was established using four different monoclonal antibodies specific for \textit{Escherichia coli} O111, \textit{E. coli} O157, \textit{Francisella tularensis} and \textit{Salmonella typhimurium} O-antigens and a panel of LPS preparations. The detection limit of antibodies was found to be 10 ng/ml, which is about a 100-fold greater sensitivity compared to conventional immunofluorescence assays. Furthermore, using LPS arrays, tularemia positive canine serum samples could be differentiated from negative samples based on the presence of significantly higher levels of anti-\textit{F. tularensis} LPS antibodies in positive samples. LPS arrays will facilitate simultaneous screening of samples against multiple antigens and are expected to find applications in diagnostics and seroepidemiology.

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1. Introduction

Gram-negative bacteria represent an important group of human and animal pathogens. These bacteria are characterized by the presence of an outer membrane with unique lipopolysaccharide (LPS) molecules. LPS is an amphipathic molecule and consists of three regions—a hydrophobic lipid-A, hydrophilic core oligosaccharide and an outer polysaccharide O-antigen. LPS plays a major role in pathogenesis (Lerouge and Vanderleyden, 2002) and the O-antigen part of LPS...
has been shown to be immunogenic (Jauho et al., 2000; Weintraub, 2003). Certain species of Gram-negative bacteria, especially *Salmonella* spp. and *Escherichia coli*, show high diversity in their O-antigen structure (Lerouge and Vanderleyden, 2002) and are identified by classic serotyping using antibodies specific for O-antigen (Poxton, 1995; Lerouge and Vanderleyden, 2002). Different serotypes within a species of Gram-negative bacteria vary in their pathogenicity and epidemiology. Certain serotypes are found to be associated with certain diseases, particularly in case of *E. coli* (Poxton, 1995). Gram-negative bacterial infections can be diagnosed by detecting antibodies specific to LPS (Poxton, 1995). Diagnosis of an infection and establishing the serotype of the bacterium involved is important not only for initiation of proper treatment but also for epidemiological studies (Poxton, 1995). Currently, clinical samples are screened for the presence of specific antibodies using immunological assays such as agglutination tests or ELISA (Jauho et al., 2000). The presence of a large number of pathogenic Gram-negative bacteria along with numerous serotypes within certain species warrants a multiplex method for rapid diagnosis of Gram-negative bacterial infections.

In the present study we tested the feasibility of using LPS arrays to detect specific antibodies to bacterial LPS. Microarray-based methods have been described for nucleic acids, proteins and recently for carbohydrates (Fukui et al., 2002; Wang et al., 2002). In addition to being multiplexed and rapid, such methods require only small quantities of samples and reagents. The LPS arrays developed were found to be specific and sensitive. The method was also used to detect the presence of specific antibodies against *Francisella tularensis* in canine serum samples.

2. Materials and methods

2.1. Lipopolysaccharides and antibodies

Lipopolysaccharide from *F. tularensis* subspecies *tularensis* was isolated using the Tri-reagent method described previously (Yi and Hackett, 2000). LPS preparations from *E. coli* O111:B4, *E. coli* O26:B6, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* serotype 10, *Salmonella enteritidis*, *Salmonella typhi-
murium*, *Shigella flexneri* serotype 1A were purchased from Sigma (St. Louis, MO). *E. coli* O157 LPS was obtained from Nacalai Tesque, Japan. Commercial preparations of monoclonal antibodies (mABs) of IgG isotype specific for *E. coli* O157, *F. tularensis* and *S. typhimurium* O-antigens were obtained from BioDesign (Saco, ME) and mABs of IgM isotype specific for *E. coli* O111 O-antigen were obtained from Abcam (Cambridge, MA). Alexa Fluor 488-labeled goat anti-mouse IgG and anti-mouse IgM antibodies (Molecular Probes, Eugene, OR) or FITC-labeled goat anti-dog IgG antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used as secondary antibodies.

2.2. Clinical samples

Five samples of canine serum (PS1 to PS5) collected from clinical cases of tularemia and positive for *F. tularensis* antibodies with known microagglutination titer (titer >20) and two canine serum samples (NS1 and NS2) negative for *F. tularensis* antibodies (microagglutination titer <20) were obtained from the laboratory of R. J. Morton.

2.3. Printing and scanning of LPS arrays

LPS arrays were printed using a microarray printer (OmniGrid, Gene Machines, CA, USA) on nitrocellulose-coated glass slides (SuperNitro microarray substrates, Telechem International, CA, USA). Each LPS sample was resuspended in buffer (Tris 20 mM, pH 7.5, NaCl 100 mM and MgCl2 5 mM) and printed with a spot spacing of 500 µm. The spot size was approximately 200 µm. For LPS immobilization studies, twofold dilutions (1 to 0.031 mg/ml) of fluorescein isothiocyanate (FITC)-conjugated *E. coli* O111:B4 LPS were printed in triplicate. For antibody binding experiments, LPS isolated from different bacteria were printed either in a twofold dilution series (1 to 0.031 mg/ml) (Fig. 2) or at a single concentration of 1 mg/ml (Figs. 3, 4, and 6) in replicates of six spots per dilution. Additionally, mouse immunoglobulins (0.1 mg/ml; Fig. 3) or canine serum (1:10 dilution; Fig. 6) were printed as positive controls. The slides were scanned using a microarray scanner (ScanArray Express, Perkin-Elmer, MA, USA), and the images analyzed using Genepix Pro 5.0 software (Axon Instruments, CA, USA).
2.4. Antibody binding experiments

The printed slides were first blocked in 2% BSA in PBS (pH 7.4) for 30 min, rinsed twice with PBS containing 0.05% Tween 20 (PBS–T) followed by a rinse in PBS and spin dried. The slides were then incubated with anti-LPS monoclonal antibodies (3 μg/ml) or serum samples (1:50 dilution) in PBS for 30 to 45 min at room temperature. After incubation, the slides were washed thrice with PBS–T for 10 min each. The slides were flooded with Alexa Fluor 488-labeled (unless otherwise specified) secondary antibodies (5 μg/ml in PBS) for 30 min and subsequently washed thrice in PBS–T followed by one wash in PBS and dried as described above. The slides were then scanned and the images were analyzed by Genepix Pro 5.0 software.

2.5. Microtiter plate-based immunoassays

Imunoassays were done as previously described (Fulop et al., 1991; Conlan et al., 2002) with modifications. Briefly, 96-well flat-bottom black microtiter plates (Microfluor1, Nalge Nunc International, Rochester, NY) were coated with LPS (10 μg/ml, 200 μl/well) in carbonate buffer, pH 9.6, at 37 °C for 3 h followed by overnight incubation at 4 °C. The LPS solution was aspirated and wells were blocked with 2% BSA in PBS at room temperature for 1 h. Wells were then washed thrice in PBS–T for five min each and incubated with different concentrations (0.001, 0.01, 0.1 and 1 μg/ml in PBS; 100 μl/well) of anti-LPS mABs in triplicate at room temperature for 1 h. After incubation the wells were washed thrice with PBS–T for 10 min each. Then, Alexa Fluor 488-labeled goat anti-mouse IgG antibodies in PBS (5 μg/ml; 100 μl/well) was added and incubated at room temperature for 1 h. Wells were washed thrice with PBS–T followed by one wash in PBS and fluorescence was recorded using a plate reader (POLARstar Optima, BMG Labtechnologies, Durham, NC).

2.6. Statistical analysis

All data presented on LPS arrays were representative of at least three independent experiments. Fluorescence signals were presented as mean±standard deviation after subtracting the background signal. In

![Fig. 1. Immobilization of bacterial LPS on nitrocellulose-coated slide. Twofold dilutions (1 to 0.03125 mg/ml) of FITC-labeled E. coli O111 LPS in buffer (Tris 20 mM, pH 7.5, NaCl 100 mM and MgCl2 5 mM) were printed in triplicate on nitrocellulose-coated slides and scanned for fluorescence (A). The slides were washed once in PBS–Tween 20 (0.05%) pH 7.4 followed by two washes in PBS alone and scanned again (B). Fluorescence signals before and after wash did not differ significantly (C and D). The fluorescence signals were normalized as ratios of the highest fluorescence value for each slide. The data points represent mean fluorescence value+S.D. of three spots for each LPS concentration (three slides).](image-url)
order to compare results from different slides, the fluorescence signals were further normalized as ratios of the fluorescence signal values obtained for mouse immunoglobulins (0.1 mg/ml; Fig. 3) or canine serum (1:10 dilution; Fig. 6) printed on the same slides as positive controls. Data were analyzed using one-way ANOVA and Tukey’s test. Means were considered significantly different if $P<0.05$.

3. Results

3.1. Immobilization of LPS on nitrocellulose-coated slides

Printing of FITC-labeled *E. coli* O111:B4 LPS on nitrocellulose-coated slides produced a concentration-dependent fluorescence signal (Fig. 1A and C). Repeated washings did not result in a significant loss of fluorescence signal (Fig. 1B and D) indicating the stable immobilization of LPS on nitrocellulose-coated

![Graph](image)

Fig. 2. Recognition of surface immobilized LPS by monoclonal antibodies. Twofold dilutions (1 mg/ml to 0.03125 mg/ml) of *F. tularensis* and *E. coli* O111 LPS were printed in replicates of six on nitrocellulose-coated slides. Slides were incubated with monoclonal antibody (3 μg/ml in PBS) specific for *F. tularensis* O-antigen. The arrays were developed with Alexa Fluor 488-labeled goat anti-mouse secondary antibodies (5 μg/ml). *F. tularensis* antibody recognized the *F. tularensis* LPS in a concentration-dependent manner. The fluorescence signals were normalized as ratios of the highest fluorescence value for each slide. The data points represent mean fluorescence value±S.D. of six replicate spots for each LPS concentration (four slides).

Fig. 3. Binding specificity of O-antigen monoclonal antibodies to LPS arrays. A panel of nine LPS preparations isolated from different bacteria was printed in replicates of six at a concentration of 1 mg/ml on nitrocellulose-coated slides. The slides were incubated with 3 μg/ml of monoclonal antibodies (IgG isotype) specific for *F. tularensis*, *S. typhimurium*, *E. coli* O157 O-antigens or monoclonal antibody (IgM isotype) specific for *E. coli* O111 O-antigen and developed with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (5 μg/ml). Antibodies specifically recognized the respective O-antigens as evidenced by the significant increase in fluorescence in each array. The fluorescence signals were normalized as ratios of fluorescence signal from mouse immunoglobulins (0.1 mg/ml) printed on the same slide as positive controls after background subtraction. The results are representative of three independent experiments. The data points represent mean±S.D. of six replicate spots for each LPS preparation.
surface without the need for any additional chemical conjugation. Also, the LPS spot morphology was found to be uniform enabling accurate quantification of the signals.

3.2. Antibody binding experiments

Monoclonal antibodies (3 μg/ml) specific for *F. tularensis* LPS successfully bound to *F. tularensis* LPS immobilized on nitrocellulose-coated slides but not *E. coli* O111 LPS (Fig. 2). The fluorescence resulting from binding of antibodies corresponded to the LPS concentrations printed. LPS concentration of 0.0625 mg/ml and above resulted in significantly higher fluorescence compared to controls. Fig. 3 shows the specific recognition of O-antigens on LPS arrays by monoclonal antibodies. Monoclonal antibodies of IgG isotype for O-antigens of *E. coli* O157, *F. tularensis* and *S. typhimurium* specifically reacted with the respective LPS but not with other lipopolysaccharides (LPSs) printed on the same slide. Similarly, monoclonal antibodies of IgM isotype specific for *E. coli* O111 O-antigen reacted only with the *E. coli* O111 LPS on arrays.

Fig. 4. Detection limit of antibodies using LPS arrays. *Francisella tularensis* and *S. typhimurium* LPS were printed at a concentration of 1 mg/ml in replicates of six on nitrocellulose-coated slides. Slides were incubated with different concentrations of monoclonal antibodies (1, 0.1, 0.01 and 0.001 μg/ml in PBS) specific for *F. tularensis* (A) or *S. typhimurium* O-antigen (B). The arrays were developed with Alexa Fluor 488-labeled goat anti-mouse secondary antibodies (5 μg/ml). The results are representative of three independent experiments. The data points represent mean±S.D. of six replicate spots for each LPS preparation.

Fig. 5. Detection limit of antibodies for microtiter assays. Microtiter plate wells were coated either with *F. tularensis* or *S. typhimurium* LPS (10 μg/ml) in carbonate buffer. Wells were incubated in triplicate with monoclonal antibodies (1, 0.1, 0.01 and 0.001 μg/ml in PBS) specific for *F. tularensis* (A) or *S. typhimurium* O-antigen (B). Binding of primary antibodies was detected using Alexa Fluor 488-labeled goat anti-mouse secondary antibodies (5 μg/ml). The results are representative of two independent experiments. The data points represent mean±S.D. of triplicate wells for each antibody concentration.
The sensitivity of LPS arrays for detection of antibodies was compared to that of conventional microtiter plate-based immunofluorescence assay using monoclonal antibodies (Figs. 4 and 5). As low as 10 ng/ml antibodies against *F. tularensis* (Fig. 4A) and *S. typhimurium* LPS (Fig. 4B) could be detected using LPS arrays. The lowest concentrations of *F. tularensis* and *S. typhimurium* mAbs that could be detected in conventional assay was 1 μg/ml (Fig. 5A and B).

LPS arrays were successfully employed to detect antibodies in clinical samples (Fig. 6). Arrays containing a panel of LPSs including *F. tularensis* LPS were exposed to 1:50 dilution of canine serum samples that tested either positive (titer >20) or negative (titer <20) for *F. tularensis* antibodies by microagglutination test. The arrays were developed with FITC-labeled anti-canine secondary antibodies. All five positive samples (PS1, PS2, PS3, PS4 and PS5) tested showed a significantly higher level of anti-*F. tularensis* LPS antibodies compared to two negative samples (NS1 and NS2; Fig. 6). Certain serum samples (PS3, PS4, NS1 and NS2) showed a slight reaction with *E. coli* O26 LPS as evidenced by the small increase in fluorescence (Fig. 6). Sample PS1 showed higher levels of antibodies against *K. pneumoniae* and *E. coli* O111 LPS (Fig. 6). This could be due to the presence of low concentrations of serum antibodies against *E. coli* and *K. pneumoniae* LPS.

4. Discussion

LPS is an important component of the outer membrane of Gram-negative bacteria. It constitutes about 10–15% of molecules in the outer membrane and covers up to 75% of Gram-negative bacterial surface (Lerouge and Vanderleyden, 2002; Caroff and Karibian, 2003). Approximately 2 million molecules of LPS are estimated to be present on the surface of *E. coli* (Raetz, 1990). The structure of lipid-A is conserved among Gram-negative bacteria, whereas

![Fig. 6. Detection of *F. tularensis* anti-LPS antibodies in serum samples. A panel of nine LPS preparations isolated from different bacteria was printed in replicates of six at a concentration of 1 mg/ml on nitrocellulose slides and incubated either with canine serum samples (dilution 1:50) positive for *F. tularensis* antibodies (microagglutination titer >20) or with canine serum samples negative (titer <20) for *F. tularensis* antibodies. Five positive and two negative samples were tested. The slides were developed with FITC-labeled goat anti-canine secondary antibodies (5 μg/ml). The level of specific *F. tularensis* antibodies were significantly higher in positive samples (PS1 to 5) compared to negative samples (NS1 and 2). The fluorescence signals were normalized as ratios of fluorescence signal from canine serum (1:10 dilution) printed on the same slide and developed with FITC-labeled anti-canine antibodies after background subtraction. The results are representative of three independent experiments. The data points represent mean ± S.D. of six replicate spots for each LPS preparation.][image]
the O-antigen is species/strain specific (Lerouge and Vanderleyden, 2002). The diversity and specificity of O-antigen is attributed to its unique sugar composition and number of possible glycosidic linkages (Lerouge and Vanderleyden, 2002; Caroff and Karibian, 2003). LPS are important antigens of Gram-negative bacteria (Jauho et al., 2000; Peula-Garcia et al., 2002) and induce specific antibody responses during infection. The antibodies directed against O-antigen component of LPS have been shown to be species/serotype specific (Jauho et al., 2000).

In the present study, we evaluated the use of LPS microarrays printed on nitrocellulose-coated slides as a platform for the detection of antibodies. The adsorption and immobilization of LPS on nitrocellulose-coated slides were found to be stable without the need for any additional chemical conjugation (Fig. 1). The presence of nitro groups renders the nitrocellulose surface hydrophobic (Wang, 2003). Binding of LPS on nitrocellulose-coated slides is likely to involve hydrophobic interaction between the lipid-A part and nitrocellulose surface. The use of nitrocellulose surface will possibly help to overcome the poor reproducibility associated with passive adsorption of LPS onto microplate surface (polystyrene/polypropylene) in ELISA (Poxton, 1995; Jauho et al., 2000).

LPS arrays printed on nitrocellulose-coated slides retained their antigenic properties as evidenced by the binding of antibodies to them (Fig. 2). The specificity of antibody binding and the ability of different antibody isotypes to bind LPS arrays were demonstrated by exposing arrays containing a number of LPSs from different bacteria on the same slide, to O-antigen specific monoclonal antibodies. Specific recognition of immobilized LPS was observed for both IgG and IgM classes of antibodies (Fig. 3). The accessibility of epitopes on immobilized LPS for IgM antibodies is important as antibodies against LPS are biased towards the IgM isotype (Trautmann et al., 1998).

The LPS arrays were capable of detecting antibody concentrations as low as 10 ng/ml (Fig. 4A and B) which is about a 100-fold improvement in sensitivity over conventional immunofluorescence assays (Fig. 5A and B) (Goldsby, 2003). It has been suggested that binding equilibrium is easily achieved with microarrays, as the binding of ligands onto a small spot of immobilized molecules does not significantly alter the concentration of ligands in solution, resulting in greater sensitivity (Wang, 2003).

Experiments were also done to test the intra- and inter-slide variability and shelf life of LPS arrays for antibody detection (data not shown). Coefficients of variation (CVs were calculated as the standard deviation of fluorescence value divided by the mean and expressed in percentage) for intra-slide and inter-slide variability were 6.98% (n=18 spots) and 16.21% (n=4 slides), respectively. In order to test the shelf-life, LPS arrays stored at room temperature for 4 weeks were compared with freshly printed arrays by simultaneously developing both the sets with primary and labeled secondary antibodies. The stored LPS arrays were found to be stable, although there was a slight decrease in the fluorescence signals compared to freshly printed slides (CV of 24.80%; n=4 slides), suggesting some degree of LPS degradation on storage.

Demonstration of anti-LPS antibodies in clinical samples is an indirect indication of infection and has been used in the diagnosis of many infections caused by Gram-negative bacteria such as Salmonella typhi (Herath, 2003), Brucella spp. (Al Dahouk et al., 2003), enterohemorrhagic E. coli O157, O26 and O111 (Ludwig et al., 2002; Tsutsumi et al., 2004), Chlamydia spp. (Brade et al., 1994), Leptospira interrogans (Priya et al., 2003), F. tularensis (Aronova and Pavlovich, 2000), Yersinia enterocolitica (Thibodeau et al., 2001), Shigella spp. (Li et al., 1993), Vibrio cholerae (Chang and Sack, 2001), P. aeruginosa (Schaad et al., 1990) and Bordetella pertussis (Trollfors et al., 2001). In this study, the suitability of LPS arrays for detection of antibodies from clinical samples was successfully demonstrated using canine serum samples. The serum samples used were known to be either positive (titer >20) or negative (titer <20) for F. tularensis antibodies based on microagglutination test. Using LPS arrays, significantly higher levels of anti-F. tularensis antibodies could be detected in all five positive samples as compared to the two negative samples (Fig. 6).

Generally, clinical samples are screened against one or two antigens chosen based on clinical symptoms and patient history, using conventional assays like agglutination tests or ELISA to arrive at a diagnosis. The LPS array approach allows rapid screening of a clinical sample against multiple Gram-negative bac-
terial infections with the possible identification of bacterial serotypes. This could become crucial to initiate an appropriate treatment. Although technically it may be possible to include most of Gram-negative bacterial LPS on a single slide, the intrinsic limitations of anti-LPS antibody detection may limit the actual number of different LPS that can be included in an array. Some bacterial LPS antigens show cross reaction with other bacterial LPS (Mitov et al., 2003) and it is not possible to diagnose all Gram-negative bacterial infections based on anti-LPS antibodies. However, inclusion of protein and carbohydrate antigens along with LPS may increase the scope of this approach to include other infectious agents.

Other potential areas of application of LPS arrays are in the field of seroepidemiology. The importance of anti-LPS antibodies has been demonstrated in the seroepidemiology of many bacterial diseases, such as V. cholerae (Suthienkul et al., 1992), Klebsiella spp. (Trautmann et al., 2004), E. coli O157 (Evans et al., 2000), typhoid fever (House et al., 2001), Chlamydia spp. (Blatz et al., 2001) and Shigella spp. (Hyams et al., 1995). LPS arrays allow simultaneous seroepidemiology of a large number of Gram-negative bacterial diseases in a population. This will help in acquisition of valuable information on disease prevalence for well-planned disease control programs.

Other promising applications of LPS arrays include evaluation of intravenous immunoglobulin (IVIG) preparations for prophylactic or therapeutic use in sepsis/endotoxemia associated with Gram-negative bacterial infections (Trautmann et al., 1998). Also, LPS arrays may be used to assess the level/quality of anti-LPS antibodies in patients with sepsis or prior to surgery, as the high level of anti-LPS antibodies is an indicator of favorable prognosis in sepsis patients (Poxton, 1995).

Microarray-based immunoassays offer several advantages over conventional immunological assays. These assays are multiplexed in nature allowing rapid and simultaneous screening of a clinical sample against multiple antigens. In addition, these assays show greater sensitivity compared to conventional assays. Apart from these advantages, microarray-based assays require only small quantities of samples and reagents.

5. Conclusions

This study demonstrates the applicability of LPS arrays for antibody detection. LPS arrays are likely to find applications in disease diagnosis, seroepidemiology, evaluation of intravenous immunoglobulin preparations and assessment of patients with sepsis/endotoxemia or prior to surgery. The major advantage of LPS arrays over conventional assays is the ability to simultaneously screen a sample against multiple antigens.

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