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Performance of a multiplexed serological microarray for the detection of antibodies against central nervous system pathogens

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A R T I C L E   I N F O

Article history:
Received 12 November 2013
Received in revised form 31 December 2013
Accepted 20 February 2014
Available online 1 March 2014

Keywords:
Microarray
Seroarray
Peptide
Recombinant protein
Glutaraldehyde

A B S T R A C T

Central nervous system (CNS) infections have multiple potential causative agents for which simultaneous pathogen screening can provide a useful tool. This study evaluated a multiplexed microarray for the simultaneous detection of antibodies against CNS pathogens. The performance of selected microarray antigens for the detection of IgG antibodies against herpes simplex virus 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), adenovirus, Mycoplasma pneumoniae and Borrelia burgdorferi sensu lato, was evaluated using serum sample panels tested with reference assays used in a routine diagnostic laboratory. The microarray sensitivity for HSV-1, HSV-2, VZV, adenovirus and M. pneumoniae ranged from 77% to 100%, and the specificity ranged from 74% to 97%. Very variable sensitivities and specificities were found for borrellial antigens of three different VlsE protein IR(6) peptide variants (IR6p1, IR6p2, IR6p4) and three recombinant decorin binding proteins A (DbpA; DbpAa, DbpA91, DbpA91G40). For single antigens, good specificity was shown for antigens of IR6p4 and DbpAa (96%), while DbpA91, IR6p1 and IR6p2 were moderately specific (88–92%). The analytical sensitivity of the microarray was dependent on the borrelial IgG concentration of the specimen. The overall performance and technical features of the platform showed that the platform supports both recombinant proteins, whole viruses and peptides as antigens. This study showed diagnostic potential for all six CNS pathogens, including Borrelia burgdorferi sensu lato, using glutaraldehyde based microarray, and further highlighted the importance of careful antigen selection and the requirement for the use of multiple borrelial antigens in order to increase specificity without a major lack of sensitivity.

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1. Introduction

One of the challenges in the laboratory diagnosis of central nervous system (CNS) infections is the relatively high number of potential causative pathogens, including e.g. herpes simplex virus 1 (HSV-1) and 2 (HSV-2), varicella zoster virus (VZV), Borrelia burgdorferi sensu lato, and Mycoplasma pneumoniae. Nucleic acid detection from cerebrospinal fluid (CSF) is usually the method of choice for the diagnosis of neuroinvasive virus infections, especially for different herpes viruses. However, virus serology from serum and CSF provides complementary information, and plays an important role particularly in prolonged infections, and in the investigation of long-term sequelae. For neuroinvasive M. pneumoniae infections, both nucleic acid detection and serology are generally used, while serology is most sensitive for neuroborreliosis. However, the serodiagnosis of Borrelia burgdorferi sensu lato is generally complicated by the very variable antibody responses observed between individuals and those at the different stages of the disease. Furthermore, the borrelial antibody response may not reflect an infection, but merely a contact with a tick carrying the pathogen. Due to the complex nature of borreliosis, several different diagnostic methods, e.g. enzyme immunoassays (EIA) and immunoblotting, are often used in parallel to obtain more specific and reliable results for diagnosis Lyme borreliosis. In addition, for the serodiagnosis of borreliosis infection, it is generally suggested that two specific antigens should react with patient’s serum to establish the diagnosis.

EIA is the most commonly used platform for the serodiagnosis of neuroinvasive infections, sometimes complemented by e.g. immunoblot. Simultaneous screening of antibodies against relevant viral and bacterial targets can provide an advantageous tool for the diagnosis of
CNS infections. Serological platforms with a multiplexed format offer a rapid, cost-effective and clinically comprehensive approach. Such platforms include multiplex bead-based immunoassays and multiple antigen microarrays, which allow for parallel antibody detection. Different multiplex bead-based immunoassays have been developed for e.g. detection of antibodies against influenza virus, Streptococcus pneumoniae, Lyme borreliosis, or pneumococcal, meningococcal and haemophilus polysaccharides, and tetanus and diphtheria toxoids (Yan et al., 2005; Yu et al., 2011; Whitelegg et al., 2012; Gerritzen and Brandt, 2012).

Multiplexed serological microarrays have been developed for e.g. simultaneous detection of HIV and its coinfections (Lochhead et al., 2011), as well as for the diagnostics of atypical pneumonia (Gouriet et al., 2008). As the detection of CNS pathogens, previous microarray designs have focused on the viral nucleic acid detection (Jääskeläinen et al., 2009). With this in mind, six antigens used for the microarray were as follows: recombinant HSV-1 glycoprotein G (gG, amino acids 84–175; Acris Antibodies GmbH, Herford, Germany), recombinant HSV-2 glycoprotein G (gG, amino acids Leu343–Asp649 fused with human superoxide dismutase (SOD); Acris Antibodies GmbH, Herford, Germany), purified whole virus antigen of attenuated adenovirus 1 (strain IA891) (HUSLAB, Finland), M. pneumoniae 1 AG (4MP67; Ani Biotech Oy, Vantaa, Finland), and recombinant VZV eG (University of Gothenburg, Gothenburg, Sweden; 11). This recombinant VZV eG antigen is produced in mammalian cells in large scale and is known to be specific for Gothenburg showing no cross-reaction with HSV (Thomsson et al., 2011; Grahn et al., 2011).

2. Materials and methods

2.1. Reference methods

HerpeSelect® HSV-1 IgG and HSV-2 IgG ELISA kits (Focus Diagnostics Inc, Cypress, CA, USA) were used for HSV antibody detection. Mycoplasma pneumoniae IgG EIA (Ani Labsystems Ltd, Vantaa, Finland) was used for M. pneumoniae antibody detection. Borrelia afzelii and VlsE IgG ELISA kit (Sekisui Virotech GmbH, Liaison® Borrelia IgG EIA (Sekisui Virotech GmbH), Porwancher et al., 2011). With this in mind, six antigens for Borrelia were selected for the microarray: three different VlsE protein IR6 peptide variants (Core Facility of Protein Chemistry, Haartman Institute, University of Helsinki, Helsinki, Finland; Sillanpää et al., 2007) of Borrelia burgdorferi sensu stricto B31 (IR6P1, Borrelia burgdorferi sensu stricto B31 (IR6P1, B. garinii IP90 (IR6P2), and B. afzelii ACAI (IR6P4), and three recombinant decorin binding proteins A (DbpA) (University of Helsinki, Helsinki; Panelius et al., 2007) derived from B. burgdorferi sensu stricto IA (DbpAa), B. garinii 40 (DbpAG40), and B. afzelii A91 (DbpA91).

Prior to spotting, HSV-1, HSV-2, M. pneumoniae, and all six borreliantigens were diluted in spotting buffer [10 mmol/L NaHCO3 (pH 9.5)] in a final concentration of 0.1 %mg/mL. VZV and adenovirus antigens were diluted in a final concentration of 1:10,000.

The 96-well polystyrene plates were activated with pre-polymerized glutaraldehyde, and each well was spotted with the 11 viral and bacterial antigens in triplicates by using the BioRobotics MicroGrid II microarray printer (BioRobotics, Cambridge, UK) as previously described (Viitala et al., 2013). Human IgG (~95% HPL purified; Sigma-Aldrich, Helsinki, Finland) was spotted in triplicates and used as a positive control on the microarray. The spotting buffer and rabbit myosin (Sigma-Aldrich) and used for a 1 h conjugation at +37 °C. 3.3’5.5’-tetramethylbenzidine liquid substrate (Sigma-Aldrich) was used for colour reaction after washing three times with PBST. After 30 min incubation at RT, the colour reaction was stopped with 100 µL of 0.5 M H2SO4. The absorbance was read at 450 nm (Multiscan RC, Labsystems, Finland).

2.2. Clinical specimens

The study material included serum samples from individual patients tested for IgG antibodies against the following antigens at the routine diagnostics of the Helsinki University Central Hospital Laboratory (HUSLAB) in Finland in 2009–2012: HSV-1 (n = 81), HSV-2 (n = 82), VZV (n = 77), adenovirus (n = 77), M. pneumoniae (n = 70), and Borrelia burgdorferi sensu lato (n = 53). The sera tested for HSV-1, HSV-2, VZV, adenovirus and M. pneumoniae IgG antibodies were deemed either positive or negative by using the respective reference methods. The sera deemed positive for Borrelia IgG (n = 35) were reactive in both two different EIA tests (Sekisui Virotech and DiaSorin) and in IgG immunoblot. The sera deemed negative for Borrelia IgG (n = 18) were non-reactive in one EIA test (Sekisui Virotech). In order to better evaluate the analytical sensitivity of the microarray, the reactive borrelial specimens were further divided into three subcategories according to their IgG antibody concentrations as follows: low IgG concentration (n = 9), intermediate IgG concentration (n = 13), and high IgG concentration (n = 13). The sum of numeric values from the two EIA tests (Sekisui Virotech and DiaSorin) was used for this categorization (low IgG concentration 25–59; intermediate 60–179; high >180).

2.3. Microarray design

Single antigens for HSV-1, HSV-2, VZV, adenovirus and M. pneumoniae were used for simultaneous screening of specific IgG-antibodies. The antigens used for the microarray were as follows: recombinant HSV-1 glycoprotein G (gG, amino acids 84–175; Acris Antibodies GmbH, Herford, Germany), recombinant HSV-2 glycoprotein G (gG, amino acids Leu343–Asp649 fused with human superoxide dismutase (SOD); Acris Antibodies GmbH, Herford, Germany), purified whole virus antigen of attenuated adenovirus 1 (strain IA891) (HUSLAB, Finland), M. pneumoniae 1 AG (4MP67; Ani Biotech Oy, Vantaa, Finland), and recombinant VZV eG (University of Gothenburg, Gothenburg, Sweden; 11). This recombinant VZV eG antigen is produced in mammalian cells in large scale and is known to be specific for Gothenburg showing no cross-reaction with HSV (Thomsson et al., 2011; Grahn et al., 2011). The complex nature of the immune response to Borrelia burgdorferi sensu lato infection requires the use of multiple serological assays to increase diagnostic sensitivity and specificity (Bacon et al., 2003; Porschuscher, 2003; Porwancher et al., 2011). With this in mind, six antigens for Borrelia were selected for the microarray: three different VlsE protein IR6 peptide variants (Core Facility of Protein Chemistry, Haartman Institute, University of Helsinki, Helsinki, Finland; Sillanpää et al., 2007) of Borrelia burgdorferi sensu stricto B31 (IR6P1, B. garinii IP90 (IR6P2), and B. afzelii ACAI (IR6P4), and three recombinant decorin binding proteins A (DbpA) (University of Helsinki, Helsinki; Panelius et al., 2007) derived from B. burgdorferi sensu stricto IA (DbpAa), B. garinii 40 (DbpAG40), and B. afzelii A91 (DbpA91).

Prior to spotting, HSV-1, HSV-2, M. pneumoniae, and all six borreliantigens were diluted in spotting buffer [10 mmol/L NaHCO3 (pH 9.5)] in a final concentration of 0.1 %mg/mL. VZV and adenovirus antigens were diluted in a final concentration of 1:10,000.

The 96-well polystyrene plates were activated with pre-polymerized glutaraldehyde, and each well was spotted with the 11 viral and bacterial antigens in triplicates by using the BioRobotics MicroGrid II microarray printer (BioRobotics, Cambridge, UK) as previously described (Viitala et al., 2013). Human IgG (~95% HPL purified; Sigma-Aldrich, Helsinki, Finland) was spotted in triplicates and used as a positive control on the microarray. The spotting buffer and rabbit myosin (Sigma-Aldrich)
were included as negative controls. In summary, there were 42 spots on each polystyrene plate well. One well was used per serum sample for sim-
ultaneous and parallel screening of different viral and bacterial antibi-
odies.

2.4. Microarray reactions

Once the antigens were spotted, the microarrays were blocked with PBS containing 2% BSA (Jackson ImmunoResearch Europe Ltd) for 30 min, followed by three washes with PBS containing 0.1% Tween 20 (PBST2). Blocked microarrays were dried, and stored at −70 °C prior to use.

Serum samples (volume 2 μl) were diluted 1:100 in 0.5% BSA (Jackson ImmunoResearch Europe Ltd) in PBS, incubated for 15 min at +37 °C on the microarrays, and washed three times with PBST2. Peroxidase conjugated AffiniPure donkey anti-human IgG (H + L) (Jackson ImmunoResearch Europe Ltd; diluted 1:5000 in PBST2) and 15 min at +37 °C on the microarrays, and washed three times with PBS containing 0.1% Tween 20 PBS containing 2% BSA (Jackson ImmunoResearch Europe Ltd) for 30 min, followed by three washes with PBS containing 0.1% Tween 20 (PBST2), subjected to 3,3′,5,5′-tetramethylbenzidine chromogen of ep(HS)TMB-mA (SDT Stereo-
pure donkey anti-human IgG (H + L) (Jackson ImmunoResearch Europe Ltd; diluted 1:5000 in PBS) was incubated on the microarrays for 15 min at +37 °C. The microarrays were washed again (3 × PBST2), subjected to 3,3′,5,5′-tetramethylbenzidine chromogen of ep(HS)TMB-mA (SDT Stereo-
specific Detection Technologies, Baesweiler, Germany) for 5 min, and dried followed by imaging.

2.5. Microarray image analysis

Images of microarrays were taken by an inverted microscope (Olympus CKX41; Olympus Finland Oy, Espoo, Finland) using Olympus DP12 digital microscope camera (Olympus Finland Oy). One microarray image consisted of the 42 spots. Spot intensities for each antigen and local background signals on the microarrays were calculated using ImageJ software (http://rsweb.nih.gov/ij/). Cut-off values were deter-
mixed for each antigen separately using five negative serum samples. Briefly, negative serum samples were screened in triplicate and analysed. The spot intensities were corrected for background signal, and cut-off values were determined for each antigen by multiplying the mean value of intensities by two. The equivocal range was set at +/−10% from the cut-off intensity value.

3. Results

The serum samples panels were tested in parallel with the serologi-
cal microarray and the reference methods. Equivocal intensity values in the microarrays were interpreted as negative throughout the calcula-
tions. Table 1 summarizes the performance of the microarray for HSV-
1, HSV-2, VZV, adenovirus, and M. pneumoniae. The sensitivity of the microarray for these targets ranged from 77% to 100%, and the specificity ranged from 74% to 97%. The overall sensitivity of the microarray for all these five targets was 91%, while the overall specificity was 85%. The best concordance as compared to reference tests was observed for HSV-1 and VZV (Table 1).

Table 2 summarizes the performance of the microarray for Lyme borreliosis. Due to the nature of microarray technique, the seroros-
dected using multiple antigens can be analysed using different rule sets to see whether some individual antigens or antigen combinations are superior over others for detecting borreliial antibodies. First, specificity and sensitivity were calculated separately for each borreliial antigen to assess their individual performance. Thereafter, the performance values were calculated for selected antigen combinations, with the require-
ment of simultaneous reactivity (sensitivity) and non-reactivity (specificity) against two different antigens. Finally, the performance was analysed for the three Borrelia species separately, with the require-
ment of reactivity against either of the two antigens specific for the species.

Good specificity was shown for IR6p4 and DbpAIa (96%), while
DbpAI91, IR6p1 and IR6p2 were moderately specific (88–92%). The speci-
ficity of DbpG40 was poor (63%), but showed the highest sensitivity (89%). The sensitivities of DbpAIa and IR6p2 were 77% and 83%, respec-
tively, and these two antigens demonstrated the best overall per-
formance. For B. burgdorferi sensu stricto, a moderate specificity (88%) and sensitivity (86%) were observed. Sensitivity for B. afzelii was moder-
ate (88%), but with a poor sensitivity (57%). For B. garinii, in contrast, sensitivity (97%) was superior of the specificity (58%). When samples positive for borreliial IgG antibodies, were divided into three subcate-
gories (low, intermediate and high), the sensitivities were more diverse between different antigens. Highest sensitivity values, ranging from 77% (DbpAI91) to 100% (DbpG40) were found among the samples showing high concentration of borreliial IgG, while the sensitivity of samples with intermediate IgG concentration varied from 23% (DbpAI91) to 85% (IR6p2). Among the low IgG concentration B. burgdorferi sensu lato samples, sensitivity levels were weak for IR6p4 (11%), DbpAI91 (11%), and IR6p1 (11%), but higher sensitivity values were found for DbpAIa (67%), IR6p2 (67%) and DbpG40 (89%) antigens.

4. Discussion

The microarray technique enables simultaneous evaluation of multi-
ple antigens, and the performance of the selected antigens can be vali-
dated using sample panels tested with selected reference assays. This study evaluated antigens for the detection of IgG antibodies against important CNS pathogens, namely HSV-1, HSV-2, VZV, adenovirus, M. pneumoniae and species of B. burgdorferi sensu stricto, which is the predominant species in North America, as well as B. garinii and B. afzelii, both predominant in Eurasia (Steere, 2001; Qiu et al., 2002; Hengge et al., 2003; Bunikis et al., 2004), causing Lyme borreliosis. Mi-
croarrays have been used for screening purposes (Bacares-Hamilton et al., 2004; Xu et al., 2008; Jääskeläinen et al., 2009; Lochhead et al., 2011; Ardizzoni et al., 2011; Viitala et al., 2013), but they can be applied also for settings requiring enhanced specificity. Recent investigations exploited the microarray platform for profiling of immune responses to Borrelia burgdorferi sensu lato (Xu et al., 2008), and another to in-
fluenza viruses, suggesting that some level of humoral immunity may not
be measured by the traditional hemagglutination inhibition assays (Koopmans et al., 2012). Recently, a similar approach was applied for the different coronaviruses (Reusken et al., 2013). We have earlier demonstrated that detection of viral antibodies and quantitation of C-reactive protein is feasible in a single microarray (Viitala et al., 2013). We have demonstrated that detection of viral antibodies and quantitation of C-reactive protein is feasible in a single microarray (Viitala et al., 2013). With the microarray technique, multiple antigens can be present in the same reaction and immunologic history can be examined simultaneously for several viral or bacterial targets. This provides an opportunity for more comprehensive and rapid identification of the causative pathogen.

The sensitivity and specificity values of the developed microarray were based on a comparison with existing reference assays. All of the reference tests in this study are being used in a large scale routine diagnostic laboratory (HUSLAB, Finland), and are considered as reliable test platforms. Some of the serum samples were reactive in the microarray test without reaction in the reference test; e.g. for HSV-1 there were 5/47, and for HSV-2 15/57 such samples. It must be noted that the specimen panels did not represent an unbiased general population, but rather individuals suspected of a particular infection. Therefore, some of these discordant samples may be truly positive for the respective targets. This is highlighted particularly in the case of adenovirus for which the microarray was reactive for all positive panel specimens (100% specificity), and for 3/19 of the negative panel specimens. The same whole adenovirus antigen was used both in the reference assay and in the microarray, and thus the superior sensitivity of the microarray platform is plausible. For the serodiagnosis of Lyme borreliosis, specificity is a critical factor, and reactions to several antigens are required to determine a history of Borrelia contact. In the microarray developed in this study, six borrelial antigens were used, and results from single antigens, as well as antigen combinations were evaluated. As demonstrated in Table 2, the requirement of simultaneous reactivity (sensitivity) and non-reactivity (specificity) against several antigens considerably decreased the performance values. Sensitivity values can be artificially increased by using less strict rules, e.g. with the requirement of reactivity for only one out of two antigens representing the same Borrelia species (Table 2). However, this will subsequently result in less optimal specificity values, and does not reflect the requirements of a diagnostic laboratory, which cannot rely on a single antigen. Our study highlighted the importance of a careful selection of diagnostic criteria and antigens when such an approach is adapted for clinical use. If the diagnostic criteria are chosen without proper validation, very variable results may be obtained when using multiple antigens.

In this study, only two antigen sites of VlsE and DbpA were used. We tested also the commercial B. burgdorferi recombinant protein p41 (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) and B. afzelii lysate (Institut Virion|Serion GmbH, Würzburg, Germany) (data not shown), but these antigens did not prove useful for the microarray platform. The DbpA sequence site has high inter- and intraspecies heterogeneity and it is considered to be a sensitive antigen in the late phase of Lyme borreliosis, and particularly in neuroborreliosis (Roberts et al., 1998; Helikä et al., 2002a,b, 2003; Schulte-Spechtel et al., 2006; Panelius et al., 2007). The use of several DbpA antigens from different species may increase specificity and sensitivity of the microarray assay. However, as DbpA is known to be more sensitive in the late phase of Lyme borreliosis, other antigens from different antigenic sites are needed to maintain a good performance of the microarray assay throughout the course of illness.

In this study we demonstrate the diagnostic potential of a multiplexed serological microarray platform for the detection CNS pathogens. In the case of B. burgdorferi sensu lato infection, specificity is the major challenge of laboratory diagnostics. This study further highlighted the requirement for the use of multiple borrelial antigens in order to increase specificity without a major lack of sensitivity. The overall performance of the platform proved promising in terms of test performance and technical features, and while it only requires a small samples volume, it is potentially adaptable also for CSF specimens.

Acknowledgements

The study was financially supported by the Finnish Funding Agency for Technology and Innovation (Helsinki, Finland), and HUSLAB (Helsinki University Central Hospital, Helsinki, Finland).

We thank Rami Lehmusto and Kirsi Aaltonen for excellent technical assistance.

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