

A protein microarray immunoassay for the serological evaluation of the antibody response in vertically transmitted infections

A. Ardizzoni · B. Capuccini · M. C. Baschieri ·
C. F. Orsi · F. Rumpianesi · S. Peppoloni · C. Cermelli ·
M. Meacci · A. Crisanti · P. Steensgaard · E. Blasi

Received: 23 February 2009 / Accepted: 15 April 2009 / Published online: 5 May 2009
© Springer-Verlag 2009

Abstract The detection of specific serum antibodies is mainly achieved by enzyme-linked immunosorbent assay (ELISA). Here, we describe the setting up of a microarray-based serological assay to screen for IgG and IgM against vertically transmitted pathogens (*Toxoplasma gondii*, rubella virus, cytomegalovirus, herpes simplex virus types 1 and 2, varicella zoster virus, *Chlamydia trachomatis*). The test, accommodated onto a restricted area of a microscope slide, consists of: (a) the immobilization of antigens and human IgG and IgM antibody dilution curves, laid down in an orderly manner; (b) addition of serum samples; (c) detection

of antigen–serum antibodies complexes by indirect immunofluorescence. The IgG and IgM curves provide an internal calibration system for the interpolation of the signals from the single antigens. The test was optimized in terms of spotting conditions and processing protocol. The detection limit was 400 fg for the IgG assay and 40 fg for the IgM assay; the analytical specificity was >98%. The clinical sensitivity returned an average value of 78%, the clinical specificity was >96%, the predictive values were >73%, and the efficiency was >88%. The results obtained make this test a promising tool, suitable for introduction in the clinical diagnostic routine of vertically transmitted infections, in parallel (and in future as an alternative) to ELISA.

A. Ardizzoni · B. Capuccini · M. C. Baschieri · C. F. Orsi ·
S. Peppoloni · C. Cermelli · E. Blasi (✉)
Dipartimento di Scienze di Sanità Pubblica,
Università di Modena e Reggio Emilia,
Via Campi, 287,
41100 Modena, Italy
e-mail: elisabetta.blasi@unimore.it

F. Rumpianesi · M. Meacci
Dipartimento Integrato dei Laboratori,
di Anatomia Patologica e di Medicina Legale,
Università di Modena e Reggio Emilia,
Modena, Italy

A. Crisanti
Dipartimento di Medicina Sperimentale e Scienze Biomediche,
Università di Perugia,
Perugia, Italy

A. Crisanti
Division of Molecular and Cell Biology,
Imperial College London,
London, UK

P. Steensgaard
Radim S.p.a.,
Pomezia, RM, Italy

Introduction

In the last few years, the development of protein microarrays has led to their application to multiple approaches: the identification of protein–protein or protein–small molecules interactions [1], cancer profiling [2], detection of microorganisms [3], drugs of abuse [4], and toxins [5] in serum and other biological fluids. To date, the field where microarray immunoassays are finding most of their applications is the identification of specific antibodies raised against allergens [6, 7], autoantigens [8], and microbial and viral pathogens [9, 10]. Recently, protein microarrays have been used to profile the humoral immune responses against a group of merozoite proteins proposed as potential candidates for a malaria vaccine [11].

The principle of microarray technology is based on the possibility for ligand-binding assays that work on a solid phase, to be miniaturized and brought into an array format [12, 13]. This concept stems from the observation that, by

accommodating onto solid support capture agents in microspots of a few microns in diameter, the ultrasensitive detection of a target analyte can be achieved by using highly specific activity labels, such as fluorophores [14]. In addition, proteins, antibodies, and other biomolecules immobilized onto solid surfaces have been shown to retain their specific binding capacity.

The present study was aimed at setting up an immunoassay, based on the protein microarray technology, for the simultaneous and quantitative determination in human serum of the antibody response against microbial and viral vertically transmitted pathogens (VTPs). Some of them are teratogenic and if the infection is contracted during the first or the last trimester of pregnancy [15], the newborn can develop more or less severe congenital syndromes and be affected by major damages to various organs and systems [16–21]; in some cases, natural abortion may occur. It follows that the early and precise serodiagnosis of these infections in the mother is of primary importance [22]. Currently, routine prenatal screening for some vertically transmitted infections (VTIs) is done during the first trimester of pregnancy in order to identify seronegative women who have to be alerted and carefully monitored because, if developing a primary infection, they are at risk of vertical transmission [16]. From here, it becomes clear the need to screen the mother for the presence of IgG and IgM antibodies against VTPs. Such screening would be costly and time-consuming if it is carried out for all of those pathogens that may seriously jeopardize the pregnancy outcome and on the entire population. Therefore, we designed a microarray containing the following antigens: *Toxoplasma gondii*, cytomegalovirus (CMV), rubella virus, herpes simplex virus types 1 and 2 (HSV1 and HSV2), varicella zoster virus (VZV), and the bacterium *Chlamydia trachomatis*, whose relevance in the vertical transmission from mother to fetus and/or newborn are well proven [23, 24]. By employing this array, the serological assay has been optimized for the evaluation of both IgG and IgM responses. Analytical parameters have been assessed and the overall performance of the microarray test has been compared to an enzyme-linked immunosorbent assay (ELISA) used as a reference test. Technical and clinical implications are discussed.

Materials and methods

Antigens

HSV1, HSV2, VZV, CMV, *T. gondii*, and *C. trachomatis* antigens were supplied by Radim S.p.a. (Rome, Italy). Rubella virus-derived antigen was purchased from Micro-

bix Biosystems (Ontario, Canada). Briefly, HSV1 McIntyre strain and HSV2 MS strain were cultivated in Hep-2 human cells. Herpes 1 and 2 antigens were prepared by sonicating the infected cells in buffer glycine, pH=9. Herpes 1 antigens were collected at a final concentration of 12.1 mg/ml. The g2 glycoprotein was purified by Herpes 2 antigen by means of lectin immunoaffinity. The final concentration was 9.5 mg/ml. VZV Ellen strain and CMV AD 169 strain were cultivated in human fibroblasts. The varicella and CMV antigens were obtained at final concentrations of 2 and 5.9 mg/ml, respectively, by sonicating the infected cells in buffer glycine, pH=10. *T. gondii* RH strain was grown in Swiss mice. The *T. gondii* antigens were obtained at a final concentration of 11.3 mg/ml, by sonicating total antigen extracts in phosphate buffer saline, pH=7.2. *C. trachomatis* LGV II-434 strain was grown in monkey kidney cells. The *C. trachomatis* antigens were obtained at a final concentration of 0.6 mg/ml, by sonicating the elementary bodies in phosphate buffer saline, pH=7.2. Rubella virus strain HPV-77 was cultivated in Vero cells. The Rubella K1S grade antigen was then obtained at a final concentration of 1.01 mg/ml, by purifying the virus suspension from the cell culture supernatant by sucrose density gradient centrifugation.

The antigens were dissolved and spotted using either of the solutions as follows: sterile distilled water (buffer 8), 1× PBS (buffer 3), 1× PBS with 400 ml/l glycerol (buffer 29), 1× PBS with 0.1 ml/l Tween 20 and 100 g/l sucrose (buffer 20), 1× PBS containing 0.5 g/l sodium dodecylsulfate and 100 g/l sucrose (buffer 21), 1× PBS with 0.1 g/l sodium dodecylsulfate (buffer 19). Sucrose and Tween 20 were purchased by Sigma Chemical Company (St. Louis, MO, USA). Sodium dodecylsulfate was provided by Fluka BioChemika (Germany). Glycerol was purchased by Incofar (Modena, Italy). 1× PBS was prepared according to the recipe described by Bacarese-Hamilton et al. [6].

Antibodies and fluorescent labels

Human IgG and human IgM were purchased from Sigma Chemical Company. Before being printed, they were serially diluted in 1× PBS containing 0.1 g/l sodium dodecylsulfate and 50 g/l sucrose (buffer 32) and in 1× PBS containing 0.1 g/l sodium dodecylsulfate (buffer 6), respectively. Anti-human IgG monoclonal antibody was provided by Radim. Anti-human IgM polyclonal antibody was purchased from Jackson ImmunoResearch Europe (Newmarket, Suffolk, UK). Anti-human IgG and anti-human IgM were fluorescently labeled by using Alexa Fluor® 555 Protein Labeling Kit (Invitrogen). The antibody labeling has been performed according to the manufacturer's instructions.

Controls

The fluorophore Alexa 555 (Invitrogen Corporation, Carlsbad, CA, USA), diluted 1:1 in 1× PBS, was employed as the signal control. Bovine serum albumin (BSA, Sigma), 10 g/l in 1× PBS, was used as the negative control. Spotting buffers without antigens and antibodies were employed as carry-over controls.

Serum samples

A panel of 74 human sera from a heterogeneous group of individuals (pregnant women, hospitalized patients, and healthy subjects) was provided by the Microbiology and Virology Service of the Policlinico Hospital in Modena. Upon collection, the 74 human sera were tested in the hospital laboratory for their IgG and/or IgM reactivity against only one or a few antigens, according to the physicians' requests. All of the sera were subsequently assessed in our laboratory by ELISA (see below) for their IgG and IgM reactivities against all of the antigens under investigation, prior to being employed in the microarray system.

ELISAs

The ELISA kits, provided by Radim (EIA Well, Radim, Rome, Italy), were used to determine: (a) the IgG reactivity against all of the antigens under investigation and (b) the IgM reactivity against CMV, *T. gondii*, rubella virus, and VZV. The IgM reactivity against HSV1 and HSV2 was evaluated by using Beia ELISA kits (Italiana Laboratori Bouty S.p.a., Milano, Italy), while the IgM reactivity against *C. trachomatis* was ascertained using Vircell ELISA kits (Granada, Spain). All of the assays were performed following the manufacturers' instructions.

Preparation of microarrays

Microbial antigens, serially diluted human IgG and human IgM, and signal and negative controls were printed onto aldehyde glass microscope slides (CEL Associates, Houston, TX, USA) using computer-controlled high-speed robotics (MicroGrid II BioRobotics, Genomic Solutions[®] Inc., Ann Arbor, MI, USA). The solutions were transferred from 384-well microtiter plates (Porvair Sciences Ltd., Shepperton, UK) onto glass slides by using stainless steel solid pins of 200 µm diameter, where each pin is estimated to transfer about 1 nl of sample onto the slide. The low-density arrays consisted of 7×7 matrices that included: (i) the seven microbial antigens printed in four replicates; (ii) the IgG and IgM antibody dilution curves in duplicate; (iii) the negative control; (iv)

the signal control; (v) the carry-over controls (blanks). Printing was performed inside a cabinet at 25°C and 55% humidity. These conditions were constantly monitored by thermohygrometers. Printed slides were stored for additional 12 h inside the cabinet prior to removal and were subsequently kept in boxes, at room temperature, in the presence of silica gel bags (Sigma Chemical Company), and used within 90 days of being printed. Figure 1a shows a schematic of the array, with the spotting concentrations of the antibody dilution curves and the seven different antigens applied in quadruplicate.

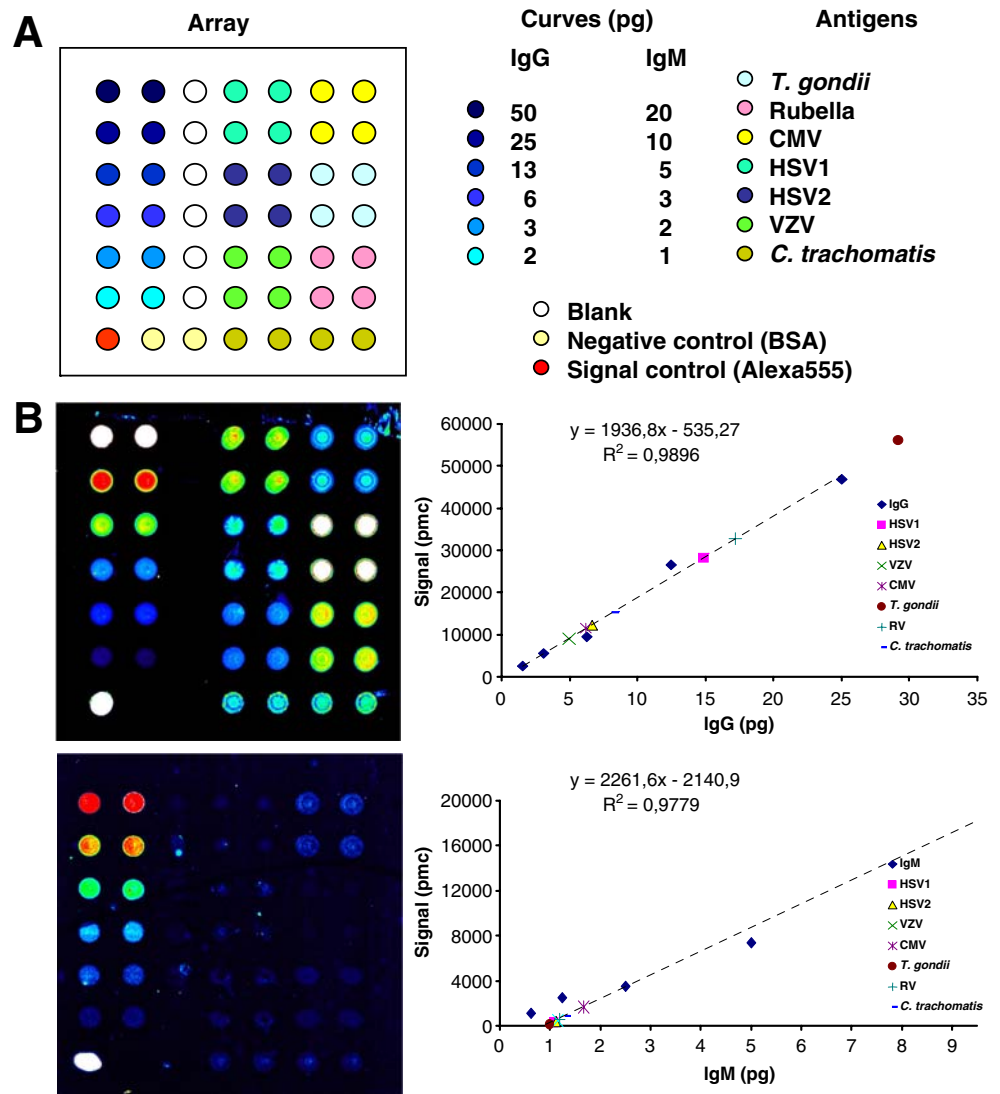
Processing of microarray slides

Printed slides were incubated for 1 h at room temperature with 200 µl of a solution containing 20 g/l BSA in 1× PBS, in order to prevent non-specific antibody binding. An adhesive square-shaped frame (Gene Frame, ABgene Limited, Epsom, UK) was stuck onto the slide surface to contain samples and reagents within the array area. Serum samples were diluted 1:200 (for the IgG test) or 1:100 (for the IgM test) in an appropriate diluent buffer (2× PBS, 10 g/l BSA, 0.1 ml/l Tween 20) and allowed to react with the array for 15 min at room temperature in a humid chamber (100 µl of diluted sample per array). Slides were washed with 1× PBS, 0.1 ml/l Tween 20. To reveal IgG bound to the printed antigens, the slides were incubated for 20 min with Alexa 555-labeled anti-human IgG monoclonal antibody diluted in the same diluent buffer employed for serum samples (see above) to a final concentration of 16 µg/ml; 100 µl of diluted anti-human IgG–Alexa 555 conjugate per array was added. To reveal IgM bound to the printed antigens, the slides were incubated for 20 min with Alexa 555-labeled anti-human IgM antibody diluted in the same diluent buffer used for the serum samples (see above) to a final concentration of 1.5 µg/ml; 100 µl of diluted anti-human IgM–Alexa 555 conjugate per array was added. Before reading the fluorescence in the scanner, the slides were washed again and dried by spinning them for 2 min in a centrifuge at 3,700 rpm at room temperature.

Data collection and analysis

The fluorescent signal from the slides was read by using a ScanArray Gx scanner (Perkin-Elmer, Cambridge, UK). The fluorescent signal from each array component was visualized in a pseudo-color scale corresponding to increasing fluorescence. Therefore, a white spot indicates a saturated signal, while spots which are colored in red, yellow, light green, and dark green, correspond to a progressively decreasing signal; blue and black spots are indicative of very low and absent signal, respectively. Images generated were saved as TIFF files and quantified with the ScanArrayExpress[™] software, provided by

Fig. 1a, b Design of the microarray immunoassay and examples of its application. **a** Scheme of the array employed. The replicates, the spotting concentrations of the antibody dilution curves, and the microbial antigens are shown. **b** Examples of processed microarrays (left) and data analysis (right). *Top* IgG test, *bottom* IgM test



Perkin-Elmer. Human IgG and human IgM dose–response curves were fitted using a Microsoft Excel linear curve fit. The amounts of IgG and IgM in the sera were determined by interpolating the photo-multiplier counts, collected at the microbial antigen spots, with the corresponding human IgG and IgM internal calibration curves.

The “analytical sensitivity” or detection limit, which indicates the smallest amount of substance in a sample that can be accurately measured by the assay, was determined by printing human IgG and human IgM dilution curves on slides in parallel with 37 replicates of BSA, taken as a non-specific protein. After being blocked, the arrays were incubated with a pool of sera, followed by either anti-human IgG or anti-human IgM, at concentrations and incubation times as per the optimized protocol (see above). The analytical sensitivity was then calculated as the mean value of the signal generated by the BSA replicates + 2 standard deviations.

The “analytical specificity” was determined by spotting slides with human IgG (50 pg), human IgM (20 pg), and human IgE (10 pg) (purchased by Calbiochem). After being blocked, the arrays were incubated with a pool of sera followed by either anti-human IgG or anti-human IgM, at concentrations and incubation times as per the optimized protocol. The specificity was calculated as the ability of each secondary antibody to bind only to its specific immobilized target antibody (i.e., IgG for the anti-human IgG and IgM for the anti-human IgM).

The interpretation of the laboratory test results was assessed according to the NCCLS guidelines [25]. Since, in most individuals, these infections may be asymptomatic, the microarray data were related to the ELISA results; therefore, the ELISA-positive subjects were considered as persons with the disease and the ELISA-negative subjects as persons without the disease. Accordingly, ELISA-related sensitivity and specificity, as well as the predictive values

and the efficiency, were evaluated. The cut-offs, determined for every antigen of the panel, were obtained from the ELISA-negative sera by calculating the average mass of antibody bound to the antigens + 2 standard deviations. The cut-off values were different for every antigen and for the two assays (IgG and IgM). As a consequence, when assessed in the microarray against each specific antigen, all of the sera with a reactivity below the cut-off value were considered to be negative, while all of the sera with a reactivity above the cut-off value were scored as positive. The ELISA-related sensitivity and specificity, the positive and negative predictive values (PPVs and NPVs, respectively), and the efficiency were then calculated according to the specifications reported by the NCCLS guidelines [25] as follows:

$$\text{ELISA – related sensitivity} = [\text{TP}/(\text{TP} + \text{FN})] \times 100$$

$$\text{ELISA – related specificity} = [\text{TN}/(\text{TN} + \text{FP})] \times 100$$

$$\text{Positive predictive value} = [\text{TP}/(\text{TP} + \text{FP})] \times 100$$

$$\text{Negative predictive value} = [\text{TN}/(\text{TN} + \text{FN})] \times 100$$

$$\text{Efficiency} = [(\text{TP} + \text{TN})/(\text{TP} + \text{FP} + \text{TN} + \text{FN})] \times 100$$

where TP = true-positives, i.e., the number of patients positive both in the microarray and ELISA; TN = true-negatives, i.e., the number of patients negative both in the microarray and ELISA; FN = false-negatives, i.e., the number of patients who were ELISA-positive and microarray-negative; FP = false-positives, i.e., the number of patients who were ELISA-negative and microarray-positive.

Results

In order to have a “gold standard” ELISA, we initially assessed the antibody reactivity of the 74 human sera against all of the antigens by using Radim ELISA kits. A summary of the ELISA results is shown in Table 1; a wide spectrum of IgG positivity was recorded for most of the antigens, with the exception of *C. trachomatis*, HSV2, and *T. gondii*, against which only four, 11, and 26 sera, respectively, resulted as reactive. In contrast, few sera were found positive in terms of IgM, the highest numbers being detected against CMV (21 out of 74). The IgM reactivity against HSV1, HSV2, and *C. trachomatis* could be assessed only by using non-Radim ELISA kits; as detailed

Table 1 Results of the enzyme-linked immunosorbent assay (ELISA) test carried out on the 74 serum samples with respect to the indicated antigens

| Antigens | Number of positive sera | | Number of negative sera | |
|-----------------------|-------------------------|--------------|-------------------------|---------------|
| | IgG | IgM | IgG | IgM |
| <i>T. gondii</i> | 26/74 | 4/74 | 48/74 | 70/74 |
| VZV | 66/74 | 3/74 | 8/74 | 71/74 |
| <i>C. trachomatis</i> | 4/74 | N.t. [2/4]* | 70/74 | N.t. [2/4]* |
| Rubella virus | 67/74 | 3/74 | 7/74 | 71/74 |
| CMV | 55/74 | 21/74 | 19/74 | 53/74 |
| HSV1 | 61/74 | N.t. [2/44]* | 13/74 | N.t. [42/44]* |
| HSV2 | 11/74 | N.t. [0/44]* | 63/74 | N.t. [44/44]* |

*The IgM reactivity against *C. trachomatis*, HSV1, and HSV2 could not be assessed (N.t. = not tested) because of the unavailability of Radim ELISA kits. In square brackets, only the results obtained by the hospital laboratory with Vircell (*C. trachomatis*) and BEIA (HSV1 and HSV2) ELISA kits on the indicated number of serum samples are reported

in brackets (Table 1), the few available data had been provided by the hospital laboratory. Thus, we proceeded by setting up the microarray test in terms of spotting conditions and processing protocol. Initially, each antigen had been diluted in 35 different buffers and spotted at ten different concentrations; the arrays were then processed with the 74 sera and the binding of serum antibodies to immobilized antigens was revealed by using fluorescently labeled anti-human IgG and anti-human IgM secondary antibodies. The results obtained in the microarray were compared to the ELISA data (data not shown). In Table 2, we summarized the microarray spotting conditions (buffer employed and antigen concentration) that more closely matched the ELISA results for each antigen and for both IgG and IgM tests. Among the several solutions assessed, buffer 29 was the most successfully utilized, often in both IgG and IgM tests (such as for HSV2 and *T. gondii*). The antigen concentrations for the IgG test varied, ranging from 1,500 µg/ml (for HSV1 and CMV) to 250 µg/ml (for rubella virus and *C. trachomatis*). For the IgM test, the antigen concentrations ranged from 750 µg/ml (for CMV) to 30 µg/ml (for VZV and *T. gondii*). Based upon these data, the arrays were designed to contain: (a) antibody curves (generated by serially diluting either human IgG or human IgM) to be used as an internal calibration system, (b) microbial and viral antigens in four replicates, and (c) signal, negative, and carry-over controls (Fig. 1a). Processing, which required only 1 h and 45 min, was performed as detailed in the Materials and methods section. The antibody dilutions provided linear dose–response curves, with coefficients of determination (r^2) ranging between 0.97 and 0.99, as shown by a representative experiment depicted in Fig. 1b.

Table 2 Spotting conditions optimized for each antigen and for both IgG and IgM microarray tests

| Antigens | IgG test | | IgM test | |
|-----------------------|-----------|------------------------------------|-----------|------------------------------------|
| | Solution | Concentration ($\mu\text{g/ml}$) | Solution | Concentration ($\mu\text{g/ml}$) |
| <i>T. gondii</i> | buffer 29 | 1,000 | buffer 29 | 30 |
| VZV | buffer 20 | 500 | buffer 3 | 30 |
| <i>C. trachomatis</i> | buffer 8 | 250 | buffer 29 | 500 |
| Rubella virus | buffer 6 | 250 | buffer 8 | 250 |
| CMV | buffer 21 | 1,500 | buffer 21 | 750 |
| HSV1 | buffer 3 | 1,500 | buffer 29 | 50 |
| HSV2 | buffer 29 | 750 | buffer 29 | 90 |

The details of the spotting solutions are listed in the [Materials and methods](#) section

Such internal calibration curves allowed us to quantitate the mass of either IgG or IgM serum antibody bound to each specific antigen by reading the signal of the antigens off the standard curve.

In order to enhance the yield of the procedure, the high-throughput robotic system was employed to accommodate multiple replicates of the array onto the same slide. As shown in Fig. 2, such a procedure allowed us to simultaneously screen six different serum samples (100 μl of diluted serum per array test, as per the conventional protocol described in the [Materials and methods](#) section) for their specific antibody reactivity. Thus, not only the laboratory devices, but also the operator-assisted steps could be appreciably reduced.

In order to establish the detection limit (analytical sensitivity) of the microarray assay, we measured the reactivity of a serum pool against a non-specific protein. Briefly, arrays consisting of BSA (37 replicates printed on the same slide) were processed with the serum pool and the fluorescently labeled secondary antibodies, anti-human IgG, or anti-human IgM. By the signal detected and the calculations performed, the analytical sensitivity was established at a value of 400 fg for the IgG test and 40 fg for the IgM test (Table 3). In order to add consistency to this data, the analytical sensitivity was also assessed by using individual serum samples. The results ranged from 80 to 720 fg (mean value 357 fg) for the IgG test and from 20 to 95 fg (mean value 51 fg) for the IgM test. The analytical specificity was also evaluated in order to demonstrate the ability of the microarray test to determine only the component it purports to measure. Specifically, the capacity of secondary antibodies to bind only to their specific targets (i.e., anti-human IgG to human IgG only and anti-human IgM to human IgM only) was evaluated. The results for the IgG test showed a specificity of 99% and more, while the IgM test returned a 99.5% specificity against IgG and a 98.5% specificity against IgE (Table 3).

In order to score the microarray results and, in turn, to evaluate the overall performance of the microarray test, we defined the cut-off values. The latter were calculated (for each antigen and for the two different tests) by averaging the results obtained in the microarray with the ELISA-negative samples; operationally, we assumed that the antibody titers of those sera were low enough to score them as negative against that specific antigen. The reactivity of each serum

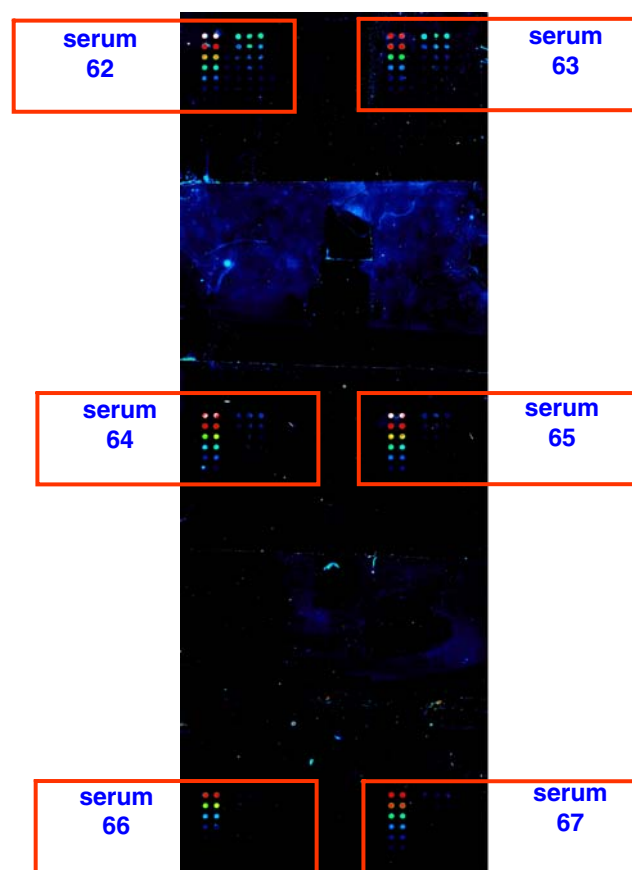


Fig. 2 Example of a multiplex microarray. Six replicates of the same array are placed on the same slide, allowing six tests to be run simultaneously

Table 3 Analytical parameters of the microarray test

| | Sensitivity ^a | Specificity ^b | |
|----------|--------------------------|--------------------------|-----------------|
| IgG test | 400 fg | 99.2% (vs. IgM) | 99.7% (vs. IgE) |
| IgM test | 40 fg | 99.5% (vs. IgG) | 98.5% (vs. IgE) |

^aThe analytical sensitivity represents the detection limit of the microarray test

^bThe analytical specificity indicates the level of discrimination of the microarray test between Igs belonging to different antibody classes

sample assessed with the microarray was then classified either positive or negative according to the presence of IgG/IgM levels above or below the cut-off values that had been defined as above. As shown in Table 4, the ELISA-related specificity of the test was very high for both IgG and IgM assays. Specifically, for the IgG assay, it ranged from 88% for *T. gondii* to 100% for three antigens (VZV, rubella virus, and CMV), and showed an overall average value of 96%. The ELISA-related specificity of the IgM assay, always greater than 90%, ranged from 94% for rubella virus to 100% for two antigens (*T. gondii* and *C. trachomatis*), with an overall average value of 98%. The ELISA-related sensitivity of the IgG test ranged from 46% for HSV2 to 97% for VZV; it was higher than 90% for three antigens and showed an overall average of 78%. The calculation of the ELISA-related sensitivity for the IgM test has not been performed yet, given the small number of IgM-positive samples for the antigens of the array chip. Finally, the PPVs and the NPVs were calculated as detailed in the [Materials and methods](#) section. As reported in Table 4, the PPVs of the IgG test ranged from 71% for HSV2 to 100% for three antigens (VZV, rubella virus, and CMV), with an overall average value of 89%; the PPVs of the IgM test were not calculated, given the low number of individuals IgM-positive for the VTPs. The NPVs of the IgG test ranged

from 33% for rubella virus to 99% for *C. trachomatis*, with an overall average value of 73%; the NPVs of the IgM test returned values ranging from 67% for *C. trachomatis* to 100% for HSV2, with an overall average value of 89%. The efficiency of both tests was very high: for the IgG assay, it ranged from 72% for HSV1 to 97% for VZV and *C. trachomatis* (overall average value: 88%); for the IgM assay, it ranged from 73% for CMV to 98% for HSV2 (overall average value: 89%).

Discussion

The search of host antibodies is one of the milestones in laboratory diagnostics. Antigen-specific antibodies raised against autoantigens, allergens, and microbial/viral antigens have served to diagnose autoimmune, allergic, or infectious diseases, respectively, as well as a means to identify candidate antigens for innovative vaccine design [6–11]. It follows that the introduction of protein/antibody microarray is a breakthrough because it allows a powerful approach for the large-scale characterization of antibody responses.

In the present work, we describe the setting up of a microarray immunoassay to be used for the clinical diagnosis of infections by a group of VTPs. We designed a microarray test which includes four classical teratogenic VTPs (*T. gondii*, rubella virus, CMV, and VZV), plus HSV1, HSV2, and *C. trachomatis*, which have controversial or no teratogenic potential, yet they can be responsible for intrauterine and/or perinatal transmission [21, 24, 26, 27]. As a first step, ELISA Radim have been chosen as gold standards and are used to assess the IgG and IgM antibody reactivity of the 74 serum samples against all of the antigens. In parallel, the microarray chips have been designed to contain the same antigen preparations of the

Table 4 ELISA-related sensitivity and specificity, positive and negative predictive values, and efficiency of the microarray tests

| Antigens | ELISA-related sensitivity (%) | ELISA-related specificity (%) | | Positive predictive values (%) | Negative predictive values (%) | | Efficiency (%) | |
|-----------------------|-------------------------------|-------------------------------|-----|--------------------------------|--------------------------------|-----|----------------|-----|
| | IgG | IgG | IgM | IgG | IgG | IgM | IgG | IgM |
| <i>T. gondii</i> | 92 | 88 | 100 | 80 | 96 | 95 | 89 | 95 |
| VZV | 97 | 100 | 99 | 100 | 78 | 97 | 97 | 96 |
| <i>C. trachomatis</i> | 75 | 99 | 100 | 75 | 99 | 67 | 97 | 75 |
| Rubella virus | 79 | 100 | 94 | 100 | 33 | 99 | 81 | 93 |
| CMV | 91 | 100 | 98 | 100 | 79 | 73 | 93 | 73 |
| HSV1 | 67 | 92 | 98 | 98 | 38 | 95 | 72 | 93 |
| HSV2 | 46 | 97 | 98 | 71 | 91 | 100 | 89 | 98 |
| Average | 78 | 96 | 98 | 89 | 73 | 89 | 88 | 89 |

Note: The ELISA-related sensitivity and the positive predictive values could not be assessed for the IgM test because of the unavailability of a sufficient number of IgM-positive samples

ELISA Radim and are processed with the same serum samples. The direct comparison of the results obtained with the two immunoassays has allowed to optimize microarray parameters, such as spotting buffer and spotting concentration, for each antigen and for both IgG and IgM tests. As a second step, the ultimate chip has been designed to contain the seven antigens (each in multiple replicates) and serial dilutions of human IgG and IgM antibodies (in duplicate), serving as an internal calibration system.

The dose–response curves generate linear trend lines with correlation coefficients close to 1, to which the signal from each single antigen can be interpolated, allowing to score every serum sample in terms of antigen-specific antibodies content. Moreover, because of the miniaturization, all of the antigens and/or antibodies of interest can be placed onto a very restricted area of a microscope slide (<1 cm²) in minute amounts (~1 nl per spot): from 1.5 ng per spot of HSV1 (for IgG test) to 0.03 ng per spot of *T. gondii* and VZV (for the IgM test). This is a particularly important feature, as both antigens and serum samples can be limited and/or expensive. Differently, in ELISA, up to 1 µg of material is needed for each well.

Taking advantage of the extreme flexibility of the robotic system, several copies of the same array can be successfully accommodated on one microscope slide (Fig. 2). Therefore, with just one processing, serial serum samples from the same individual can be run in order to have a one-test kinetic of the antibody profile. Correspondingly, sera from clusters of different patients sharing clinical/diagnostic problems can be tested in one shot. Thus, the protein microarray is a unique opportunity, since it may investigate large numbers of serological parameters at once and in just 1 h and 45 min. Differently, ELISA requires (at least) two and half hours and, more important, supplies results for just one parameter at a time. Taken together, miniaturization and multiparametricity make the microarray a very interesting tool to be used as an assay in parallel with (and in future as an alternative to) ELISA in clinical laboratories.

Upon assessment of the analytical parameters, we found that the IgM microarray test displays interesting results concerning analytical sensitivity (0.04 pg) and analytical specificity (>98%); the results of the IgG assay show an even higher analytical specificity (>99%) and an analytical sensitivity of 0.4 pg. This, ultimately, means that the microarray test is sensitive enough to detect up to 400 fg of antigen-specific IgG and 40 fg of antigen-specific IgM bound to the immobilized corresponding antigens. Concerning the overall performance of the microarray serological assay, the ELISA-related sensitivity returns an average value close to 80% for the IgG. Furthermore, the ELISA-related specificity is $\geq 90\%$ for all of the antigens and for the IgG and IgM tests. According to these findings, the test described here is particularly suitable for the

assessment of primary infections during pregnancy, when the highest possible specificity should be achieved. A false-positive result may have very important consequences, such as psychic or economic trauma for the mother, unsuitable therapeutic protocols harmful for the fetus, and, eventually, the decision of a therapeutic abortion [15, 25].

In order to evaluate the probability of the microarray immunoassay to provide correct diagnostic results, the predictive values have been determined. They are a *posteriori* assessment and indicate which are the probabilities for a positive or negative result to be a true result. This ultimately depends not only on the test itself, but also on the prevalence of the infection among the population included in the study: a high incidence of the infection will contribute to generate high PPVs, whereas a low incidence will result in low PPVs and high NPVs [25, 28]. Accordingly, in our study, very high PPVs have been obtained for the IgG test against VZV, rubella virus, and HSV1, which are common pathogens [29–31]; oppositely, the highest NPVs have been recorded for HSV2 and *C. trachomatis*, which are known to be uncommon [31–33]. Finally, the high values of efficiency obtained for IgG and IgM tests further underlines the ability of the microarray serological assay to provide true results, either positive or negative [25].

An immunoassay like the one proposed here would suit the demand not only for a routine antenatal screening against potentially dangerous pathogens, but also for a pre-pregnancy assessment of the females' immune status. In case of seronegative individuals, a prophylactic (where available) intervention may be applied (i.e., vaccination against rubella and VZV); alternatively, if the pregnancy has already started, a primary infection may be spotted very early and a suitable therapeutic approach can be promptly applied. In addition, the mother's antibody profile can be strictly monitored throughout the pregnancy, providing diagnostic results against many pathogens in one test. The microarray system described here can also be used for monitoring patients who are at high risk of developing these very same diseases, because they have some sort of immunodeficiency due to natural (HIV infection, hematological malignancies) or artificially induced causes (transplant recipients). Furthermore, it should be noted that the seven-antigens platform described here can be easily enlarged and/or modified, allowing to screen the antibody profiling against a well-defined group of antigens, depending upon the clinical issue that needs to be addressed at the bench level.

Acknowledgments We thank Radim S.p.a. for the financial support. The authors would like to acknowledge Dr. Giuseppina Gallucci, from Radim S.p.a., for having selected and provided all of the antigens included in this study. Special thanks go to the non-profit foundation "Fondazione Cassa di Risparmio di Modena" for their generous economical contribution, which allowed us to purchase the robotic arrayer and the scanner to read the processed microarrays.

References

- MacBeath G, Schreiber SL (2000) Printing proteins as microarrays for high-throughput function determination. *Science* 289:1760–1763
- Nam MJ, Madoz-Gurpide J, Wang H, Lescure P, Schmalbach CE, Zhao R, Misek DE, Kuick R, Brenner DE, Hanash SM (2003) Molecular profiling of the immune response in colon cancer using protein microarrays: occurrence of autoantibodies to ubiquitin C-terminal hydrolase L3. *Proteomics* 3:2108–2115. doi:10.1002/pmic.200300594
- Cai HY, Lu L, Muckle CA, Prescott JF, Chen S (2005) Development of a novel protein microarray method for serotyping *Salmonella enterica* strains. *J Clin Microbiol* 43:3427–3430. doi:10.1128/JCM.43.7.3427-3430.2005
- Du H, Wu M, Yang W, Yuan G, Sun Y, Lu Y, Zhao S, Du Q, Wang J, Yang S, Pan M, Lu Y, Wang S, Cheng J (2005) Development of miniaturized competitive immunoassays on a protein chip as a screening tool for drugs. *Clin Chem* 51:368–375. doi:10.1373/clinchem.2004.036665
- Rucker VC, Havenstrite KL, Herr AE (2005) Antibody microarrays for native toxin detection. *Anal Biochem* 339:262–270. doi:10.1016/j.ab.2005.01.030
- Bacarese-Hamilton T, Mezzasoma L, Ingham C, Ardizzoni A, Rossi R, Bistoni F, Crisanti A (2002) Detection of allergen-specific IgE on microarrays by use of signal amplification techniques. *Clin Chem* 48:1367–1370
- Bacarese-Hamilton T, Gray J, Ardizzoni A, Crisanti A (2005) Allergen microarrays. *Methods Mol Med* 114:195–207
- Joos TO, Schrenk M, Höpfl P, Kröger K, Chowdhury U, Stoll D, Schörner D, Dürr M, Herick K, Rupp S, Sohn K, Hämmerle H (2000) A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. *Electrophoresis* 21:2641–2650. doi:10.1002/1522-2683(20000701)21:13%3C2641::AID-ELPS2641%3E3.3.CO%3B2-X
- Bacarese-Hamilton T, Ardizzoni A, Gray J, Crisanti A (2004) Protein arrays for serodiagnosis of disease. *Methods Mol Biol* 264:271–283
- Bacarese-Hamilton T, Mezzasoma L, Ardizzoni A, Bistoni F, Crisanti A (2004) Serodiagnosis of infectious diseases with antigen microarrays. *J Appl Microbiol* 96:10–17. doi:10.1046/j.1365-2672.2003.02111.x
- Gray JC, Corran PH, Mangia E, Gaunt MW, Li Q, Tetteh KKA, Polley SD, Conway DJ, Holder AA, Bacarese-Hamilton T, Riley EM, Crisanti A (2007) Profiling the antibody immune response against blood stage malaria vaccine candidates. *Clin Chem* 53:1244–1253. doi:10.1373/clinchem.2006.081695
- Ekins RP, Chu FW, Biggart E (1990) Multispot, multianalyte, immunoassay. *Ann Biol Clin (Paris)* 48:655–666
- Ekins RP, Chu FW (1992) Multianalyte microspot immunoassay. The microanalytical “compact disk” of the future. *Ann Biol Clin (Paris)* 50:337–353
- Ekins RP, Chu FW (1999) Microarrays: their origins and applications. *Trends Biotechnol* 17:217–218. doi:10.1016/S0167-7799(99)01329-3
- Gilbert GL (2002) Infections in pregnant women. *Med J Aust* 176:229–236
- Gerber S, Hohlfield P (2003) Screening for infectious diseases. *Childs Nerv Syst* 19:429–432. doi:10.1007/s00381-003-0766-5
- Enders G, Miller E, Cradock-Watson J, Bolley I, Ridehalgh M (1994) Consequences of varicella and herpes zoster in pregnancy: prospective study of 1739 cases. *Lancet* 343:1548–1551. doi:10.1016/S0140-6736(94)92943-2
- Montoya JG, Remington JS (2008) Management of *Toxoplasma gondii* infection during pregnancy. *Clin Infect Dis* 47:554–566. doi:10.1086/590149
- Dontigny L, Arsenault MY, Martel MJ, Biringer A, Cormier J, Delaney M, Gleason T, Leduc D, Martel MJ, Penava D, Polsky J, Roggensack A, Rowntree C, Wilson AK; Society of Obstetricians and Gynecologists of Canada (2008) Rubella in pregnancy. *J Obstet Gynaecol Can* 30:152–168
- Engman ML, Malm G, Engstrom L, Petersson K, Karltorp E, Tear Fahnehjelm K, Uhlen I, Guthenberg C, Lewensohn-Fuchs I (2008) Congenital CMV infection: prevalence in newborns and the impact on hearing deficit. *Scand J Infect Dis* 40:935–942. doi:10.1080/00365540802308431
- Kriebs JM (2008) Understanding herpes simplex virus: transmission, diagnosis, and considerations in pregnancy management. *J Midwifery Womens Health* 53:202–208. doi:10.1016/j.jmwh.2008.01.010
- Stegmann BJ, Carey JC (2002) TORCH infections. Toxoplasmosis, other (syphilis, varicella-zoster, parvovirus B19), rubella, cytomegalovirus (CMV), and herpes infections. *Curr Womens Health Rep* 2:253–258
- Darville T (2005) *Chlamydia trachomatis* infections in neonates and young children. *Semin Pediatr Infect Dis* 16:235–244. doi:10.1053/j.spid.2005.06.004
- Ngassa PC, Egbe JA (1994) Maternal genital *Chlamydia trachomatis* infection and the risk of preterm labor. *Int J Gynaecol Obstet* 47:241–246. doi:10.1016/0020-7292(94)90568-1
- Hannon WH, Atkinson MA, Ball DJ, Goldsmith ML, Matsson PNJ, Whitley RJ (2001) Specifications for immunological testing for infectious diseases; approved guideline—second edition. Clinical and Laboratory Standards Institute (formerly NCCLS), vol 21, pp 1–42
- Enright AM, Prober CG (2002) Neonatal herpes infection: diagnosis, treatment and prevention. *Semin Neonatol* 7:283–291
- Thorp JM Jr, Katz VL, Fowler LJ, Kurtzman JT, Bowes WA Jr (1989) Fetal death from chlamydial infection across intact amniotic membranes. *Am J Obstet Gynecol* 161:1245–1246
- Altman DG, Bland JM (1994) Diagnostic tests 2: predictive values. *BMJ* 309:102
- Gabutti G, Rota MC, Guido M, De Donno A, Bella A, Ciofi Degli Atti ML, Crovari P; Seroepidemiology Group (2008) The epidemiology of Varicella Zoster virus infection in Italy. *BMC Public Health* 8:372–380. doi:10.1186/1471-2458-8-372
- Rota MC, Bella A, Gabutti G, Giambi C, Filia A, Guido M, De Donno A, Crovari P, Ciofi Degli Atti ML; Serological Study Group (2007) Rubella seroprofile of the Italian population: an 8-year comparison. *Epidemiol Infect* 135:555–562. doi:10.1017/S0950268806007400
- Suligoi B, Torri A, Grilli G, Tanzi E, Palù G; The Italian Herpes Management Forum (2004) Seroprevalence and seroincidence of herpes simplex virus type 1 and herpes simplex virus type 2 infections in a cohort of adolescents in Italy. *Sex Transm Dis* 31:608–610. doi:10.1097/01.olq.0000140013.60224.ba
- Pasquini P, Mele A, Franco E, Ippolito G, Svennerholm B (1988) Prevalence of herpes simplex virus type 2 antibodies in selected population groups in Italy. *Eur J Clin Microbiol Infect Dis* 7:54–56. doi:10.1007/BF01962174
- Latino MA, Bello L, Lanza A, Leotta E, Tersiev P, De Intinis G, Spagnolo E, Smirne C, Grieco R (2002) *Chlamydia trachomatis* infection among sexually active young women in Italy. *Sex Transm Infect* 78:305–307. doi:10.1136/sti.78.4.305