The protein "mycoarray": a novel serological assay for the laboratory diagnosis of primitive endemic mycoses

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SUMMARY

A protein microarray containing fungal antigens (the "mycoarray") has been set up to provide rapid and appropriate serodiagnosis of primitive endemic mycoses, an important cause of morbidity and mortality in an increasingly high number of patients. The mycoarray consists of three antigen extracts (histoplasmin, coccidioidin and Coccidioides "TP") and antibody dilution curves were spotted on microarray slides. The arrays were processed with coccidioidomycosis and histoplasmosis patients' sera or with control sera and the occurring immunocomplexes were detected by indirect immunofluorescence. In agreement with clinical and microbiological diagnosis, the results distinguished between histoplasmosis and coccidioidomycosis patients. In addition, the assay could clearly discriminate between IgM and IgG antibody reactivity. No reactivity was ever observed in the arrays processed with negative control sera. Therefore, this pilot study demonstrates that the "mycoarray" is sensitive and specific enough to discriminate between healthy individuals and patients with histoplasmosis or coccidioidomycosis. Because of miniaturization and multiparametricity, the new assay cuts costs and processing time. Thus, once clinically validated and implemented as a large-scale array, the "mycoarray" will be ready to be applied to the daily clinical practice.

KEY WORDS: Primitive endemic mycoses, Protein microarray, Serology, *Coccidioides immitis*, *Histoplasma capsulatum*

INTRODUCTION

Primitive endemic mycoses traditionally include histoplasmosis, coccidioidomycosis, blastomycosis and paracoccidioidomycosis.
Coccidioidomycosis, caused by *Coccidioides immitis*, is mainly found in the South-Western USA, where the incidence of the infection has increased 4-fold in the last 10 years (Parish and Blair, 2008). The peculiar climatic conditions of these areas, along with natural forces (dust storms, earth-

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quakes, etc.) and the anthropogenic disturbance of the soil, are having a great impact on the arthroconidia dispersion (Comrie, 2005) and the increased incidence of infection. Interestingly, certain ethnic groups have been shown to be more at risk of developing the disease (Crum *et al.*, 2004). Histoplasmosis, caused by *Histoplasma capsulatum*, is the most common and world-wide diffused primitive mycosis. Besides of being scattered in microfoci in Asia and Africa, where *H. capsulatum var duboisii* is well known, *H. capsulatum var capsulatum* (Kauffman, 2007) is highly endemic in the mid-Western and South-Eastern USA (Guimarães *et al.*, 2006). Also in Europe, *H. capsulatum* has been described, although its endemicity and/or imported origin remains to be established (Kauffman, 2007). Overall, this pathogen is the main responsible for

respiratory primitive fungal disease(s): histo-plasmosis affects healthy individuals and, more importantly, immunocompromised patients, where it may occur either as primary infection following direct exposure to the pathogen or as reactivation of a latent infection, acquired years before (Ashbee *et al.*, 2008). The majority of infected individuals develop a mild subclinical infection, which often remains undiagnosed. When symptomatic, the pulmonary infection may range from an influenza-like illness to a progressive pneumonia, which evolves as a chronic cavitary pulmonary disease or even as a highly lethal disseminated infection, especially in immunocompromised patients, including individuals with AIDS (Goodwin Jr. *et al.*, 1981; Kauffman, 2007) and patients receiving potent immunomodulators (Deepe, 2007).

Given the lack of specific clinical manifestations in immunocompetent subjects and the severity of the diseases in susceptible hosts, a quick and early laboratory diagnosis is crucial in order to initiate a suitable therapeutic protocol (Yeo and Wong, 2002). In particular, because of their naturally low frequency rate, clinical cases occurring outside the endemic areas are often diagnosed only at late stages of the disease. A common feature of primitive mycoses, such as coccidioidomycosis and histoplasmosis, is their capacity to elicit a strong humoral immune response; therefore, the detection of specific serum antibodies can be considered a relevant diagnostic parameter (Laniado-Laborín, 2007). For example, the identification of anti-*Coccidioides* IgM or IgG antibodies is particularly useful in the diagnosis of coccidioidomycosis, also because of the lack of other rapid diagnostic approaches and the limitations of the culture methods (Wheat, 2006a). By conventional assays, such as complement fixation (CF), double immunodiffusion (ID) and enzyme immunoassays (EIA), anti-*Coccidioides* and anti-*Histoplasma* antibodies may be detected (Wheat, 2003). Nevertheless, each of these methods suffers from one or more drawbacks: ID provides only qualitative results (Galgiani, 1992); both ID and CF suffer from low sensitivity (Wheat, 2007), whereas EIAs are more sensitive, but less specific, especially in endemic areas (Wheat, 2006b). Lastly, they all take a long time to provide definitive results, especially ID and CF (Pappagianis and Zimmer, 1990).

Recently, the development of protein microarray technology, a miniaturization of ligand-binding assays brought into an array format (Ekins *et al.*, 1990), has led to multiple applications both in research and diagnostics. Specifically, microarray immunoassays have been described for the detection of specific antibody profiles in allergies (Bacarese-Hamilton *et al.*, 2002), autoimmune disorders (Joos *et al.*, 2000) and infectious diseases, including mycoses (Bacarese-Hamilton *et al.*, 2004; Ardizzoni *et al.*, 2009; Mochon *et al.*, 2010), thus providing novel and promising diagnostic tools.

This paper describes the development of a novel serological test, the protein "mycoarray", for the diagnosis of primitive endemic mycoses, namely histoplasmosis and coccidioidomycosis. The "mycoarray" is a serological assay built up on a protein microarray platform. It is based on a chip containing 3 relevant fungal extracts (*Coccidioides immitis* "TP" antigen, coccidioidin and histoplasmin) along with IgM and IgG dilution curves, used as internal calibration system. The protein "mycoarray" has been proven to discriminate healthy individuals from patients with histoplasmosis or coccidioidomycosis, according to their humoral immune responses.

MATERIALS AND METHODS

Antigens
Antigens were supplied by Meridian Bioscience Europe S.r.l (Milano, Italy): 2 different formulations of *Coccidioides immitis*, the early antigen (*Coccidioides* "TP") and the late and highly immunogenic antigen (coccidioidin or *Coccidioides* "F", a purified cultural filtrate at concentration of 100 units/ml); histoplasmin, a purified mycelial cultural filtrate from the growth of *Histoplasma capsulatum*, containing both "H" and "M" antigens.

Antibodies and fluorescent labels
Human IgM and IgG were purchased from Sigma Chemical Company (St. Louis, MO, USA). Before being printed, the IgM antibodies were diluted in 1X PBS containing 0.1 g/l sodium dodecylsulphate, and the IgG in 1X PBS containing 0.1 g/l sodium dodecysulphate and 50 g/l sucrose. Anti-human IgM polyclonal antibody was purchased

from Jackson Immuno Research Europe (Newmarket, Suffolk, UK). Anti-human IgG monoclonal antibody was provided by Radim S.p.a. (Roma, Italy). Secondary antibodies were fluorescently labelled with Alexa Fluor 555 Protein Labeling Kit (Invitrogen). Purified goat IgG (Pierce) and Alexa 647-labelled anti-goat IgG (Invitrogen) were used for the initial optimization of the array's spotting conditions.

Serum samples
Six serum samples, collected in Northern Italy from patients with primitive mycoses, were em-

ployed. Details concerning each patient's clinical history are given in table 1. All sera were previously tested both for the anti-*C. immitis* and anti-*H.capsulatum* antibodies using the double diffusion test according with the Oudin and Outcherlony technique (Fungal Immunodiffusion System, Meridian Bioscience Europe S.r.l.). Control sera, collected from 15 healthy donors, were run in parallel. Goat reference serum samples, positive and negative for the 3 antigens, were purchased by Meridian Bioscience Europe S.r.l. and used for the initial optimization of the array's spotting conditions.

TABLE 1 - *Clinical and laboratory data relative to the 6 patients included in the study.*

Patient	Case-history	Haematological parameters	X-ray or computed tomography	Clinical symptoms	Serology (ID)	Culture	Histology	Clinical diagnosis	Therapy
1 Italian, Man	Onset 30 days after returning from Arizona, USA	C Reactive Protein increased, hypereosinophilia	Chest nodular lesions	Present	Anti-coccidioidin antibodies	*C. Inmitis* in BAL	*Coccidioides* BAL positive	Invasive coccidioido-mycosis	Itraconazole
2 Italian, Man	Onset 20 days after returning from Otonga, Ecuador	C Reactive Protein increased	Chest nodular lesions	Present	Anti *H. capsulatum* ("M" band) antibodies	Not available	Not done	Invasive histoplasmosis	Itraconazole
3 Italian, Man	Onset 30 days after returning from Otonga, Ecuador	C Reactive Protein and AST/ALT increased	Chest nodular lesions	Present	Anti *H. capsulatum* ("M" band) antibodies	Not available	Not done	Invasive histoplasmosis	None
4 Italian, Man	Immuno-suppressed	Not available	Not available	Present	Anti *H. capsulatum* ("M" band) antibodies	*H. capsulatum* in blood	Not available	Invasive histoplasmosis	Liposomal Amphotericin B
5 Ivorian, Man	HIV+	Not available	Abdominal adenopathy	Initially present, later healed	Anti *H. capsulatum* ("M" band) antibodies	Not available	Not available	Invasive histoplasmosis	Liposomal Amphotericin B
6 Liberian, Man	HIV+, Kaposi's sarcome	CD4 counts: 20 cells/µl; viral load: 1455308 copies/ml; pancytopenia: 1020 WBC	Chest and CNS nodular lesions	Present	Not assessed	Not available	*Histoplasma* bone marrow positive	Invasive histoplasmosis	Ambisone then Fungizone

Preparation of microarrays

Fungal antigens, serially diluted human antibodies (IgM and IgG) and carry-over 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., MI, USA). Briefly, the solutions were transferred from 384-well microtiter plates (Porvair Sciences Ltd., Shepperton, UK) onto glass slides 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 7x7 matrices that included:

1) the 3 fungal antigens printed in 4 replicates;
2) human IgM and human IgG antibody dilution curves in duplicate;
3) negative controls;
4) signal controls;
5) carry-over controls (blanks).

Printing was performed inside a cabinet at 25°C and 55% humidity. These conditions were constanly monitored by thermohygrometers. Printed slides were kept for an additional 12 hours inside the cabinet prior to removal and were subsequently stored in boxes, at room temperature, in presence of silica gel bags (Sigma Chemical Company) and used within 90 days of being printed.

Processing of microarray slides

Printed slides were incubated for 1 hour at room temperature with 200 µl of a solution containing 20 g/l BSA in 1X 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. The serum samples were diluted 1:100 (for the IgM test) and 1:200 (for the IgG test) in an appropriate diluent buffer (2X PBS, 10 g/l BSA, 0.1 ml/l Tween 20) and allowed to react with the array for 15 minutes at room temperature in a humid chamber (100 µl of diluted sample per array). Slides were washed with 1X PBS, 0.1 ml/l Tween 20. To reveal IgM or IgG bound to the printed antigens, the slides were incubated for 20 minutes with Alexa 555-labelled anti-human IgM or with Alexa 555-labelled anti-human IgG; the secondary antibodies were diluted in the same diluent buffer employed for serum samples (see

above) to a final concentration of 1.5 µg/ml (anti-human IgM) and 6 µg/ml (anti-human IgG); 100 µl of diluted secondary antibody per array was added. Before reading the fluorescence in the scanner, the slides were washed again and dried by spinning them for 2 minutes in a centrifuge at 3700 rpm at room temperature.

Data collection and analysis

The fluorescent signal was detected by reading the slides in a ScanArray Gx scanner (Perkin-Elmer, Cambridge, UK). The signal from each array component was visualized in a pseudo-color scale corresponding to increasing fluorescence, where a white spot indicates a saturated signal and spots which are coloured in red, yellow, light green, 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 Perkin-Elmer.

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

In order to define the serological results obtained with the microarray system, cut-off values were determined for all the antigens of the panel, by calculating the average mass of antibody bound to the antigens + 2 standard deviations of the 15 negative control sera. The cut-off values were different for every antigen and for the 2 assays (IgG and IgM). Therefore, all the sera with a reactivity below the cut-off value were considered negative, while all the sera with a reactivity above the cut-off value were scored as positive.

RESULTS

Initial experiments were carried out to set up the spotting conditions and processing protocol to be used in the "mycoarray" assay, namely to establish the best condition for each antigen to form immunocomplexes with its specific antibodies. In particular, histoplasmin, coccidioidin and *Coccidioides* "TP" antigen were spotted undiluted

and serially diluted, both in distilled water and in PBS. The chip was then processed with goat reference positive and negative sera, followed by fluorophore-labelled anti-goat IgG. These optimization experiments allowed us to establish that the array's best performance was achieved when histoplasmin and *Coccidioides* "TP" antigens were spotted undiluted, and coccidioidin was diluted 1:10 in PBS (data not shown).

In order to assess anti-*Histoplasma* and anti-*Coccidioides* antibodies in human sera, the arrays, generated as above, were processed with sera from patients affected by coccidioidomycosis or histoplasmosis and then exposed to Alexa 555-labelled anti-human IgM or anti-human IgG.

Figure 1 shows 4 representative images of arrays processed with sera from patients with coccidioidomycosis (top arrays) or histoplasmosis (bottom arrays). The following results were observed. First, the test showed antibody-class specificity: the arrays processed with anti-human IgM returned a fluorescent signal only from the IgM calibration curves (Figure 1A, blue frames); similarly, the arrays processed with anti-human IgG returned a fluorescent signal only from the IgG calibration curve (Figure 1B, red frames). Second, both *Coccidioides* antigens (Figure 1A, top array, green frames) revealed high IgM reactivity in the serum from the patient with coccidioidomycosis, whereas no reactivity was ob-
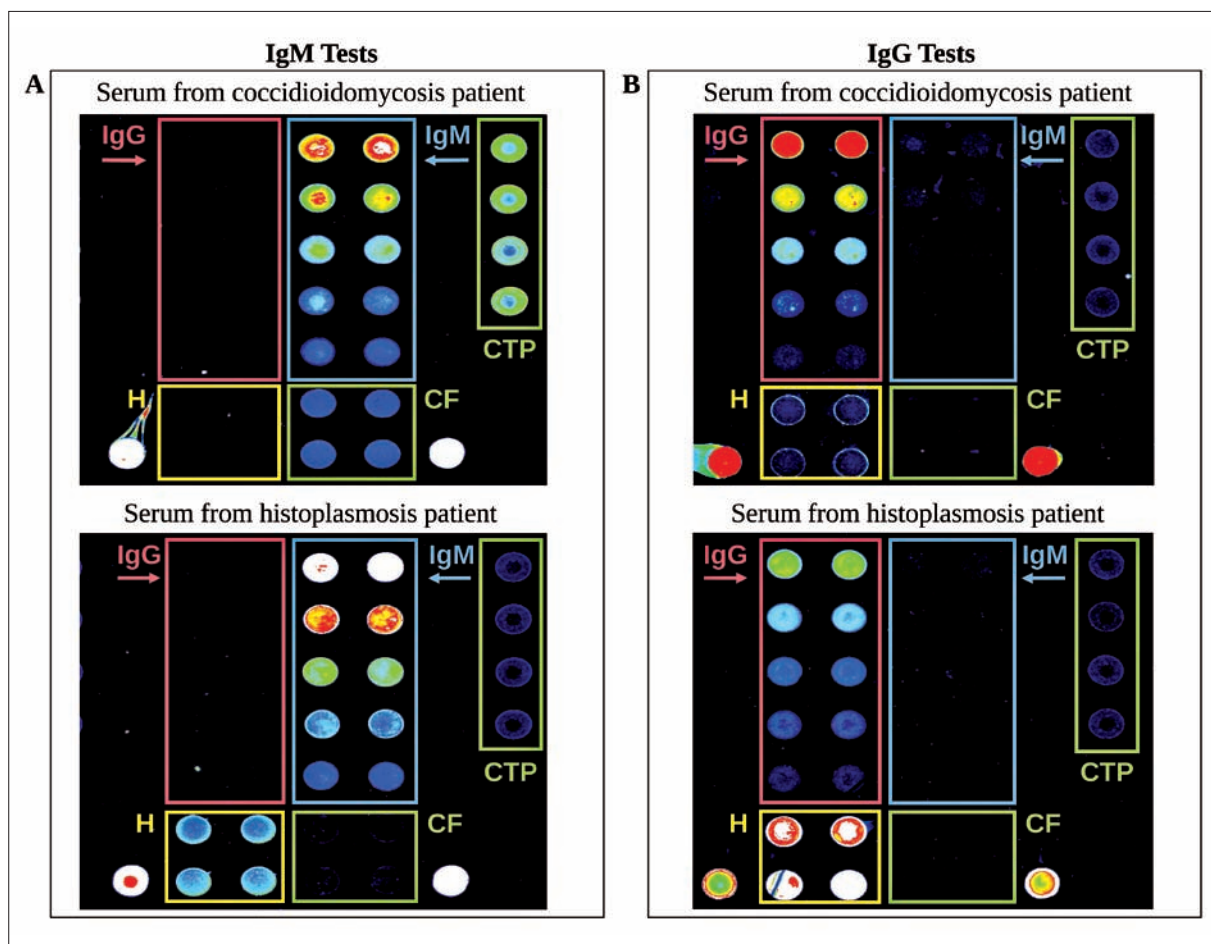

FIGURE 1 - *A: Examples of IgM reactivity against Coccidioides immitis (top array) and Histoplasma capsulatum (bottom array) as assessed by "mycoarray". B: Examples of IgG reactivity against Coccidioides immitis (top array) and Histoplasma capsulatum (bottom array) as assessed by "mycoarray". IgM dilution curves are surrounded by blue frames; IgG dilution curves are surrounded by red frames; green and yellow frames indicate Coccidioides antigens and histoplasmin respectively.*

served in this same patient from histoplasmin (Figure 1A, top array, yellow frame). Third, upon assessment of IgG reactivity on this same serum sample, no detectable signal was recorded from the *Coccidioides* antigens (Figure 1B, top array, green frames). Fourth, the serum from the histoplasmosis patient showed high and specific reactivity only against the histoplasmin spots, both in terms of IgM (Figure 1A, bottom array, yellow frame) and IgG (Figure 1B, bottom array, yellow frame) antibodies.

Using the "mycoarray" assay, 6 serum samples from patients with histoplasmosis or coccidioidomycosis, recently diagnosed in Northern Italy, and 15 serum samples from healthy indi-

viduals were assessed. As summarized in table 1, out of the 6 patients, 3 were travellers (patients 1-3), 1 was pharmacologically immunosuppressed (patient 4) and 2 were HIV-positive (patients 5 and 6). Laboratory data and clinical features led to definitive diagnosis of invasive mycoses.

Figure 2 shows quantitative results obtained on the 6 patients by "mycoarray". For each fungal antigen and for both the IgM (left panels) and IgG (right panels) tests, the cut-off values (vertical lines) were calculated as detailed in Materials and Methods. As shown in figures 2A and 2C, the IgM reactivity against Coccidioidin and *Coccidioides* "TP" respectively was detected only in serum 1. Moreover, histoplasmin specific IgM
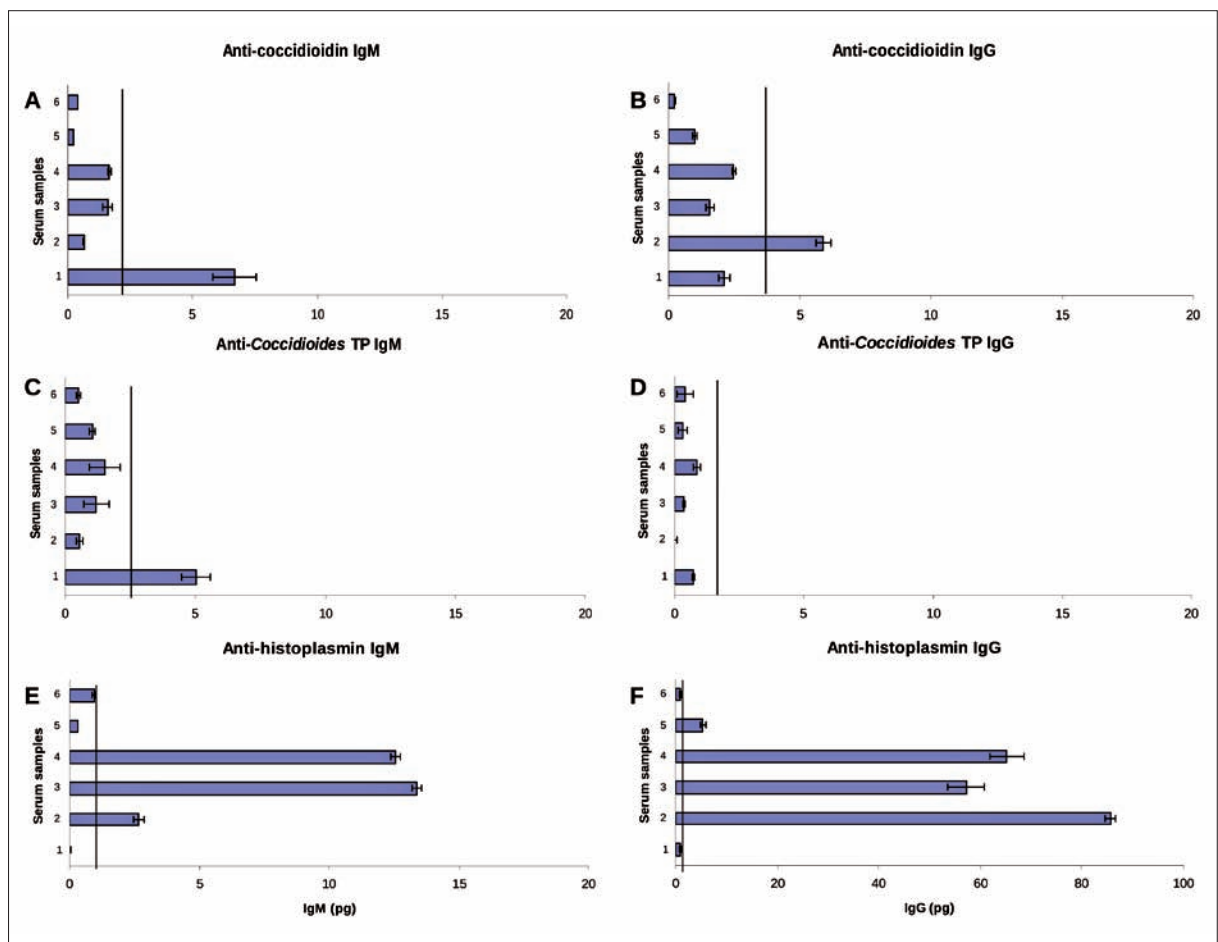

FIGURE 2 - *Serological reactivity of 6 serum samples collected from patients affected by primitive mycoses, as assessed by "mycoarray". A, B: IgM and IgG antibodies against coccidioidin; C, D: IgM and IgG antibodies against Coccidioides TP antigen; E, F: IgM and IgG antibodies against histoplasmin. Vertical lines represent the cut-off limits. The horizontal columns depict the mass of bound antibody and the bars indicate the standard deviations (four replicates of two independent experiments).*

antibodies were detected in sera 2, 3 and 4 (Figure 2E). Samples 5 and 6 were IgM negative for all the antigens (Figure 2A, 2C and 2E). Concerning the IgG test, no sera showed any IgG reactivity against coccidioidin and *Coccidioides* TP antigen (Figure 2B and 2D), except for serum 2 that showed IgG antibodies against-coccidioidin (Figure 2B). Furthermore, histoplasmin provided clear IgG positive results in all the sera, except for sera 1 and 6 (Figure 2F). No antibody reactivity against any of the fungal antigens was ever detected in the 15 negative control sera from healthy donors, either in the IgM or in the IgG test (data not shown).

Table 2 summarizes the clinical diagnosis and serological results obtained by the "mycoarray". On the one hand, the patient with coccidioidomycosis (patient 1) was identified as positive in terms of IgM reactivity, by both the coccidioidin and the *Coccidioides* TP antigen, whereas no reactivity was detected in the IgG test. In addition, no reactivity was detected against histoplasmin in this same patient. On the other hand, when the sera from patients with histoplasmosis were considered (patients 2-6), the "mycoarray" revealed IgM reactivity in sera 2, 3 and 4. These same sera were also highly positive in terms of anti-*Histoplasma* IgG response; moreover, significant levels of IgG were revealed in all the other samples from histoplasmosis patients, with the exception of serum 6 that showed no antibody reactivity at all.

DISCUSSION

Coccidioidomycosis and histoplasmosis are primitive mycoses still characterized by a strong endemic feature; yet, the increased frequency of worldwide travel and immigration are transferring the incidence of clinical cases outside endemic areas. Notwithstanding the occurrence of several imported cases of primitive mycoses recently diagnosed in Northern Italy, the incidence of these infections is often underestimated (Farina *et al.*, 2005; Galetta *et al.*, 2007). This is mainly due to the still low frequency of clinical cases recorded in this country, together with several drawbacks of the available diagnostic methods (Pappagianis and Zimmer, 1990; Galgiani, 1992; Wheat, 2006b; Wheat, 2007).

This paper describes the "mycoarray", a novel laboratory tool that couples the usefulness of serological investigations in the diagnosis of endemic mycoses with the advantages of the protein microarray technology. The array, containing 2 different antigens from *C. immitis* (coccidioidin and *Coccidioides* TP antigen) and an antigenic preparation from *H. capsulatum* (histoplasmin), has been used to assess serum samples from 6 patients clinically diagnosed with invasive mycoses, as documented by ID assay, conventional culture procedures and/or histology. As detailed above, when assessing serum from patient 1, who had been hospitalized for invasive coccidioidomycosis (Rossanese A *et al.* 11th Conference of the

TABLE 2 - *Summary of the 6 patients' serological reactivity against fungal antigens, as assessed by "mycoarray".*

Clinical diagnosis	Serum sample	IgM response to			IgG response to		
		Cocc[a]	*TP*	*Histo*	*Cocc*	*TP*	*Histo*
Coccidioidomycosis	1	++	+	-	-	-	-
Histoplasmosis	2	-	-	+	+	-	++++
"	3	-	-	+++	-	-	+++
"	4	-	-	+++	-	-	+++
"	5	-	-	-	-	-	+
"	6	-	-	-	-	-	-

aCocc = coccidioidin; TP = Coccidioides TP antigen; Histo = histoplasmin.

International Society of Travel Medicine, Budapest, Hungary, May 24-28, 2009, Abstract PO06.22), the assay reveals a clear IgM response (well above the cut-off values) against *Coccidioides TP* and a much higher signal against coccidioidin. Notoriously, when an infection by *C. immitis* is contracted, the first detectable antibodies are IgM against "TP", which appear within several days from the infection and gradually wane away within a few months; the subsequent IgG response appears within 1 to 3 months, persists for long time and is mainly directed against the F antigens (coccidioidin), known to be highly immunogenic (Smith *et al.*, 1956; Sawaki *et al.*, 1966; Bronnimann and Galgiani, 1989; Cox, 1989; Pappagianis and Zimmer, 1990; Anstead and Graybill, 2006). The present results obtained with the "mycoarray" show that TP and coccidioidin are both effective in detecting IgM response, namely both antigens are good "capture" systems in revealing IgM positive individuals (Figure 2A and 2C).

In contrast, when evaluating the IgG reactivity, neither coccidioidin nor TP reveal any positivity in patient 1, which indicates that he is experiencing a very recently acquired infection. Moreover, the lack of IgM reactivity against coccidioidin and *Coccidioides* "TP" in all the other patients' sera, as well as in all the healthy control subjects (data not shown), demonstrate the specificity of the assay. Furthermore, histoplasmin allows detection of IgM reactivity in sera 2, 3 and 4, implying that histoplasmosis has recently been acquired by these patients. Interestingly, the ID assay in these patients provided IgM negative results (no "H" bands). Our findings imply a better performance of the "mycoarray" in terms of enhanced sensitivity with respect to the conventional serological assays. In contrast, patients 5 and 6 show no IgM reactivity (Figure 2E); these data may be related to either no recent contact with the pathogen or to hyporesponsiveness of the host.

Concerning the IgG test, no reactivity was detectable against both *C. immitis* antigens (Figure 2B and 2D) with the exception of anti-coccidioidin IgGs observed in serum 2 (Figure 2B). This serum was collected from a patient clinically diagnosed for histoplasmosis. Accordingly, the "mycoarray" has revealed high IgM/IgG reactivity against histoplasmin. The unexpected presence of anti-coccidioidin antibodies in patient 2 may be interpreted in two different ways. First, they may represent a long-lasting memory of a previous infection; this is in line with the lack of IgM and with a report by Cox (1989), which described the persistency of anti-coccidioidin IgG for long times after the infection/disease; second, the anti-coccidioidin reactivity may be a false-positive result due to a cross-reaction phenomenon, already described (Cox, 1979). While we tend to exclude this last hypothesis, since none of the other sera from histoplasmosis patients show any positivity for *Coccidioides* antigens, we rather propose the "mycoarray" as a sensitive tool, capable of providing a very informative picture of the clinical history of an individual by a careful evaluation of his antibody repertoire.

As regards anti-*Histoplasma* IgG reactivity, anti-histoplasmin IgG antibody titers were detectable in patients 2-5 (Figure 2F), in line with the results of the ID test, where clear "M" bands were observed (Table 1). The negative results observed in patient 6 are likely related to his AIDS condition: no antibody response is detectable, likely because his immune system is deeply hampered by the HIV infection. Taken together, our results point out to the "mycoarray" as a more sensitive diagnostic tool for the serology of primitive endemic mycoses.

Protein microarrays enable sensitive and specific detection of serum antibodies (Joos *et al.*, 2000; Bacarese-Hamilton *et al.*, 2002; Bacarese-Hamilton *et al.*, 2004; Ardizzoni *et al.*, 2009; Mochon *et al.*, 2010). In particular, quantitative evaluation of specific IgG and IgM against pathogens responsible for vertically transmitted infections has recently been described (Ardizzoni *et al.*, 2009). The main advantages of serological screening by microarray are multiparametricity and miniaturization. The multiparametricity implies deposition onto the same microscope slide, of an enormous number of determinants, by spotting many antigens from different pathogens, different antigenic preparations from the same microbial agents, and many replicates of the same antigen. The miniaturization allows a reduction in costs (minimal amounts of antigens, serum samples and reagents) as well as in timing. Here, we demonstrate that the "mycoarray" assay allows identification of *Histoplasma*- and *Coccidioides*-positive subjects, according to their

serological response. Although aware of the limitations of the present study (3 fungal antigens, 6 patients), we provide initial evidence on the usefulness of the "mycoarray" in clinical mycology laboratories since, unlike conventional tests, it allows low reagents/sera consumption in parallel with rapid, one-shot and multiple determinations. These features may become particularly important in non-endemic countries where infected subjects are often diagnosed at later stages of the diseases. By employing an assay such as the "mycoarray", these fungal infections may be spotted earlier, even before disease onset.

In conclusion, we have described a novel serological assay sensitive, specific, rapid and multiparametric, deserving consideration also for future applications, such as:

1) its enlargement by addition of other serologically relevant fungal antigens;

2) inclusion of antibodies as "capture molecules" for specific fungal antigens that will make this test suitable also for diagnosis during the initial phases of infection, when antigenemia is a most valuable diagnostic parameter;

3) use of highly sensitive detection systems, such as the tyramide amplification already successfully applied to the allergen microarrays (Bacarese-Hamilton *et al.*, 2002), which may facilitate serological analysis, especially for critical patients where antibody titers are very low.

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