Journal of Microbiological Methods 149 (2018) 9–13

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Semiquantitative Dot Blot with the GRA8 antigen to differentiate the stages of toxoplasmosis infection

Juan Gabriel Costa[a,*], María Julia Variliño[b]

[a] Cátedra de Fisicoquímica, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Santa Fe, Santa Fe, Argentina
[b] Hospital Escuela Eva Perón, Av. San Martín 1645, Granadero Baigorria, Santa Fe, Argentina

ARTICLE INFO

Keywords:
Dot Blot
Toxoplasmosis
Diagnosis
Acute infection
GRA8
P35

ABSTRACT

In this work we present a novel methodology to differentiate the phases of toxoplasmosis infection: the "semiquantitative Dot Blot". It is a simple technique that does not require expensive equipment, does not involve a long technique development, and can be used in a low-complexity laboratory. In this study, two recombinant sequences of *Toxoplasma gondii* GRA8 antigen were used, and specific IgG antibodies were detected in selected patient samples. This method makes it possible to obtain a score for each serum and define whether the patient is in the acute or chronic phase of the infection.

The sensitivity and specificity results varied depending on the antigenic sequence used. With GRA8A, 62.1% and 72.7% were obtained, while with GRA8B, 82.8% and 72.1% were obtained, respectively. Although the sensitivity and specificity values were not close to 100%, they were similar to those reported with the same antigens in ELISA. Therefore, this quantitative technique would be a good alternative to ELISA.

1. Introduction

Toxoplasma gondii is a protozoa parasite that infects all mammals and birds, producing toxoplasmosis. Worldwide, the prevalence rate of *T. gondii* infection varies between countries and affects from 10 to 80% of the population (Robert-Gangneux and Darde, 2012). Fortunately, toxoplasmosis is generally asymptomatic and causes a self-limiting disease in humans. However, the acute phase of the infection acquired during gestation can be transmitted to the fetus (congenital infection) and may cause miscarriage, permanent neurological damage, visual impairment, or other malformations in the newborn (Costa, 2017; Peng et al., 2011).

Toxoplasmosis acquired during gestation represents a difficult scenario for the clinician due to its subclinical course in most pregnant women and the unpredictable long-term outcomes of congenital infection. To implement suitable therapies in an appropriate time frame and to avoid neonatal malformations, it is essential to interpret the infection phase correctly (Li et al., 2000a; Villena et al., 1998). Yet, discordance between diagnostics is common, despite the availability of a great variety of tests.

Detection of toxoplasma infection and differentiation between the acute and chronic phases are mainly based on serological tests that recognize anti-toxoplasma antibodies in blood. One of the most common procedures is the ELISA (Enzyme-Linked Immunosorbent Assay). It is a simple test for a research and diagnostic laboratory, many samples can be processed at the same time, and many variants are available (Aubert et al., 2000; Costa and Duré, 2016a). However, new procedures should be sought so as to make the test accessible to low-complexity diagnostic centres, which would not need to use expensive instruments or perform numerous assays to confirm the diagnosis. Therefore, many researchers are still exploring new techniques or variants of those currently used (Costa and Variliño, 2018; Kotresha and Noordin, 2010).

The techniques with nitrocellulose membrane support are a possible solution to this problem, such as Dot-Blot or Dot-ELISA. They generate a colored and insoluble precipitate that allows a direct visual reading of the results (Capobiango et al., 2016; Pappas et al., 1983) and they are versatile techniques that may be applied to the diagnosis of many samples. These immunoassays have a better capacity to adsorb proteins and they are cheaper, faster, and easier than the classic ELISA (Agudelo-Flórez and Palacio, 2009; Huang et al., 2004; Lyashchenko et al., 2000). For this reason, several laboratories develop new diagnostic tests on nitrocellulose membrane supports (Khammari et al., 2014; Sithigorngul et al., 2011).

Abbreviations: T. gondii, Toxoplasma gondii; ELISA, Enzyme-Linked Immunosorbent Assay; Ag, Antigen; Ab, Antibody; ISAGA, Immunosorbent agglutination; HAI, Hemagglutination assay; NIS, Negative infection sera; CIS, Chronic infection sera; AIS, Acute infection sera; PIS, Positive infection sera; PCR, Polymerase chain reaction; ROC, Receiver Operating Characteristic; CI, Confidence interval

* Corresponding author.
E-mail address: jcosta@fbcb.unl.edu.ar (J.G. Costa).

https://doi.org/10.1016/j.mimet.2018.04.015
Received 14 March 2018; Received in revised form 16 April 2018; Accepted 17 April 2018
Available online 21 April 2018

In turn for diagnostic purposes, one of the most promising acute phase-specific antigens is GRA8 (also called P35) (Costa and Duré, 2016b; Costa and Vilariño, 2018). This protein is secreted from the dense granule, an organelle of *Toxoplasma gondii*. The GRA proteins allow the survival of the parasite within the host cell. GRA8 is among the antigens (Ags) for which there is consensus about their effectiveness in phase differentiation (Babaie et al., 2011; Costa and Vilariño, 2018; Hiszczyjska-Sawicka et al., 2005; Li et al., 2000b; Lu et al., 2006).

In this paper, we present a new method to differentiate the phases of toxoplasmosis infection, the "semiquantitative Dot Blot". We detected specific IgG antibodies and used two recombinant sequences from GRA8 antigen. Semiquantitative Dot Blot is a modification of the regular Dot Blot (Pappas et al., 1983) but in our technique a serum sample is exposed to several dilutions of the same antigen and then a score is calculated based on the reactions that it generates in them. The final score defines if the serum belongs to the acute phase or the chronic phase of the infection. In this work, two different sequences of the recombinant GRA8 antigen were tested with the same technique. Both sequences had shown good results in ELISAs in previous studies (Costa and Duré, 2016b).

2. Materials and methods

2.1. Samples

Serum samples were obtained from three Argentine health centres, where the patients' blood was taken. The blood was clotted (30 minutes at 37 °C) and then centrifuged at 1600*g* for 10 min (minutes) in the same centres. The supernatant, serum, was collected and stored at −80 °C. The samples were collected from adults between 2009 and 2011. We classified the samples into groups of sera according to the results obtained with the commercial tests: IgG avidity test (VIDAS Toxo IgG Avidity, bioMerieux, Marcy lÉtoile, France), IgG indirect immunofluorescence (IFI, in-house test), Sabin-Feldman dye test (assay in-house test), IgM and IgA immunosorbent agglutination (ISAGA, bioMérieux, Marcy lÉtoile, France), hemagglutination assay (HAI, Toxo test HAI Wiener Lab, Rosario, Argentina), and anti-*T. gondii*-IgG ELISA (Toxoplasma IgG ELISA kit, Sigma-Aldrich, St. Louis, U.S.A.). All employed sera were subjected to 3 to 6 assays to confirm by several commercial tests the serological group that the individual belonged to. Four serological groups were formed:

Negative infection sera (NIS): 10 sera from people without toxoplasmosis. These were evaluated with IgM ISAGA and also with at least two of the following techniques: IgG ELISA, IFI, Sabin-Feldman dye test, and/or HAI. Sera were negative in all these techniques.

Chronic infection sera (CIS): 43 sera from people with the chronic phase of toxoplasmosis infection. These were tested with at least two of the following techniques: IgG ELISA, IFI, Sabin-Feldman dye test, and/or HAI. Sera were positive in these techniques, but sera were negative in IgM ISAGA and had a high avidity index in the avidity assay.

Acute infection sera (AIS): 29 sera from people with recently acquired (acute phase) toxoplasmosis. These were evaluated with at least two of the following techniques: IgG ELISA, IFI, Sabin-Feldman dye test, and/or HAI. Sera were positive in these techniques. Sera were also positive in IgM and IgA ISAGA and they had low avidity in avidity assay.

Positive infection sera (PIS): sera from individuals with toxoplasmosis. This group consisted of combined AIS and CIS.

2.2. Cloning and expression of antigens

Two *Toxoplasma gondii* GRA8 sequences were cloned: GRA8A and GRA8B. The primers used for amplification of these sequences by PCR (polymerase chain reaction) were as follows:

- GRA8A-FW: 5′-GAATTCATGGCTTTACCATTGCGTG-3′
- GRA8A-RV: 5′-AAGCTTCGTTGGCGGGGGATGCTG-3′
- GRA8B-FW: 5′-GAATTCGGAATGCCCAAGCCAGAG-3′
- GRA8B-RV: 5′-AAGCTTTGGAGTGCCCACTGGATACG-3′

GRA8A and GRA8B were cloned into the pET32a vectors (Novagen, Darmstadt, German). All sequences were introduced into the BL21 (DE3) strain of *Escherichia coli*. The bacteria were cultured in Luria-Bertani medium at 37 °C until they reached an optic density between 0.4 and 0.5 (read in spectrophotometers, MRClab, Jinjia village, China). Antigen expression was induced for 3 h at 37 °C with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (Promega, Madison, U.S.A.).

The TrxA fusion protein (which the pET32a vector automatically adheres to the cloned antigens) was also separately expressed with the procedure just described.

2.3. Purification

The culture medium with the bacteria (which contained the expressed antigen) was centrifuged at 2000*g* for 10 min. The bacterial pellet was isolated, suspended in binding buffer (20 mmol/L imidazole, 0.3 mol/L NaCl, and 0.05 mol/L NaH_2PO_4), broken by sonication using an ultrasonic disintegrator, and centrifuged. Ag was purified from solution with a nickel pseudo-affinity IDA-Sepharose column (Ni-NTA, Invitrogen, Carlsbad, U.S.A.). The elution solutions were: 0.3 mol/L NaCl and 0.05 mol/L NaH_2PO_4, with 50, 100, 250, and 500 mmol/L imidazole.

Protein concentration and purity were evaluated by 15% polyacrylamide gel electrophoresis under denaturing conditions, as published by Schägger and von Jagow (1987).

2.4. Semiquantitative Dot Blot

It was carried out in the same way as a regular Dot Blot on a nitrocellulose membrane strip (GE Healthcare, Amersham Hybond-ECL, Little Chalfont, United Kingdom), but the same antigen was applied onto each strip in 6 different dots (volume per dot: 1 μL) in decreasing concentrations: 0.7, 0.35, 0.14, 0.07, 0.014, and 0.007 mg/mL (for GRA8A and GRA8B, separately). Then the dots were allowed to dry. The strip was completely immersed in blocking solution (5% non-fat skim milk in PBS consisting of 137 mmol/L NaCl, 2.7 mol/L KCl, 10 mol/L Na2HPO4, and 1.8 mmol/L KH2PO4, pH 7.4) and then incubated for 60 min. It was washed three times by complete immersion in PBS-tween (PBS with 0.05% tween 20) for 1 min every time. The strip was immersed in 1/100 diluted serum in PBS-milk (1% non-fat skim milk in PBS) and incubated for 45 min. The membrane was washed three times with PBS-tween as described above. Human IgG was detected using IgG anti-human goat antibody (Ab) conjugated with horseradish peroxidase (Abcam, Cambridge, United Kingdom). The strip was completely immersed in Ab conjugate diluted 1/1500 in PBS-milk, and incubated for 45 min. It was washed three times again with PBS-tween. Finally, the blot reaction was revealed by immersing the membrane completely in the chromogen (3, 3′-diaminobenzidine; Sigma-Aldrich, St. Louis, U.S.A.) for 15 min.

Each strip was analysed in duplicate and was read blindly by two independent observers at the same time. The observers always agreed on their assessment.

Score calculation: Each serum was exposed to a strip. Up to six color dots could appear on each strip. In the development, a score was assigned to each dot that appeared: 4 when it was very intense; 2 when it had medium or low intensity; and 1 when the dot did not give color. Finally, the scores were multiplied to obtain only one score per serum/strip.

The GRA8A, GRA8B, and TrxA proteins were previously evaluated in a traditional Dot Blot. The technique was developed as described for semiquantitative Dot Blot but using a single dot (instead of six) with a protein concentration of 0.3 mg/mL.