Standardization of a latex agglutination test for coproantigen detection of *Fasciola* sp. in bovine cattle stool

Lina Paola Orejarena Ávila^{1,2} · Erika Marcela Inguilan Benavides^{1,2} ·
Leonardo Padilla Sanabria^{1,2} · Delia Piedad Recalde-Reyes^{1,2} ·
Carlos Andrés Rodríguez-Salazar^{1,2} · Jhon Carlos Castaño-Osorio^{1,2}

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Abstract Fasciolosis is a zoonotic parasitic disease, which affects humans and animals; diagnosed through noncommercial immunoassay tests that cannot be used on the field. Thereby, establishing the optimal conditions to develop a latex agglutination technique with IgG and IgM antibodies directed against excretion/secretion antigens of *Fasciola* sp. is a priority. Latex particles were sensitized with IgG and IgM antibodies directed against excretion/secretion antigens of *Fasciola* sp. The specificity of the antibodies was determined against antigens of different helminths and protozoa; the sensitivity and specificity of the test was evaluated against a previously standardized direct ELISA. The coupling rates of the IgG and IgM antibodies were 85.77 and 100%, respectively. The minimum detectable concentration of *Fasciola* sp. excretion/secretion antigens, diluted in a phosphate-buffered saline, was 1.589 mg/mL(IgG) and 0.158 mg/mL(IgM) and for the antigens incorporated in the bovine cattle stool it was 3.178 mg/mL(IgG) and 1.589 mg/mL(IgM). The test showed crossed reaction against *Giardia* sp., and *Cryptosporidium* sp. antigens. Agreement of the IgG and IgM latex test against the ELISA test was of 78.78 and 96.96%, respectively; the specificity found was of 100% for both tests and sensitivity was 78.79% (IgG) and 96.97% (IgM). This work standardized the latex agglutination technique to detect *Fasciola* sp. antigens in bovine cattle stool.

Keywords Fasciolosis · Agglutination · Latex · ELISA · Antigens · Monoclonal antibodies

Introduction

Fasciolosis is a zoonotic parasitic disease caused by the trematode species *Fasciola hepatica* and *Fasciola gigantica*. It affects humans and animals, generating liver damage, especially in the bile conducts; it is acquired through the intake of metacercariae, which are the infective phase of the parasite, which can be found in some raw vegetables or in water (Valero et al. 2012). Regarding the rate of fasciolosis infection, the World Health Organization (WHO) in 2014 indicated that the number of people infected throughout the world is at least 2.65 million, distributed in Africa, Europe, Asia, and Latin America; with this last region being the most affected (Anuracpreeda et al. 2016; Valero et al. 2012).

In Colombia, this parasitosis has increased during the last 25 years, given that climate change has favored the proliferation of its host (an air-breathing snail from the *Lymnaea* family) to new microenvironments, consequently, bringing the proliferation of the parasite (Uribe et al. 2014). All this added to the difficulty in diagnosing, especially during the pre-patent and patent phases of the disease, where the clinical signs are not noticeable, which is why detection of parasitic antigens is a diagnostic alternative (Recalde-Reyes et al. 2014).

Currently, the definitive diagnosis to detect this parasite is observation of its eggs in fecal matter, through optical microscopy, using concentration techniques. Said technique is inconvenient because it requires trained personnel in the preparation and visualization of the samples; besides, the elimination of eggs in large amounts occurs when the

✉ Delia Piedad Recalde-Reyes
drecalde@uniquindio.edu.co

¹ Grupo Inmunología Molecular (Gymol), Universidad del Quindío, Armenia, Quindío, Colombia

² Facultad Ciencias de la Salud, Universidad del Quindío, Carrera 15 Calle 12 Norte, Armenia, Quindío, Colombia

disease is in an advanced stage and the hepatic damage can be irreversible (Gajewska et al. 2005; Valero et al. 2012). In addition, ELISA techniques have been developed that permit detecting coproantigens during the first weeks of infection, like the ELISA Fascidig[®] (Espino and Finlay 1994) developed in the Pedro Kouri Institute of Tropical Medicine in Cuba, or the ELISA using mAb MM3 from the Parasitology Laboratory at Universidad Santiago de Compostela, Spain (Valero et al. 2009). Said techniques show high sensitivity and specificity, but are not commercial and require medium complexity equipment and processes that cannot be brought to the field.

Due to the difficulty of early diagnosis of fasciolosis, this work sought to standardize a rapid latex agglutination technique for coproantigen detection of *Fasciola* sp., in bovine cattle stool.

Materials and methods

Production of the polyclonal mouse IgM (pAb IgM) antibody

The study used *Fasciola* sp. excretion/secretion antigen to immunize a male, *Mus musculus*, mouse, BALB/C strain, 6 months old raised under sanitary conditions and adequate feeding regime.

Prior to immunizing the mouse, pre-immune serum was obtained; thereafter, it was inoculated via intraperitoneal, 100 µL of *Fasciola* sp. ES/Ag, at a concentration of 600 µg/mL plus Freund's complete adjuvant (Sigma, USA) (proportion 1:1 v/v). Seven days later, it was sacrificed, through cervical dislocation, obtaining 700 µL of total blood through cardiac puncture, which was centrifuged at 800×g, during 10 min at 4 °C. The plasma obtained was separated and stored at − 20 °C.

Obtaining excretion/secretion antigens from *Fasciola* sp. (*Fasciola* sp. E/S Ag)

Adult *Fasciola* sp. were extracted from the bile conducts of livers of infected bovine cattle. The livers were donated by the Frigocafe S.A. slaughterhouse. The parasites extracted were washed five times with phosphate-buffered saline (PBS) 20 mM pH 7.0 preheated at 37 °C and deposited in 24-well culture boxes CytoOne[®] 1 parasite per well with RPMI 1640 medium + L-Glutamine 200 mM (Gibco) + Antibiotic/Antimycotic (200 units of penicillin, 200 µg streptomycin, 0.25 µg amphotericin B) (Gibco) under sterility conditions and incubated at 37 °C and 5% CO₂ environment during 12–16 h; once transpired, the medium was collected, centrifuged at 12,000×g at 4 °C for 10 min, filtered through a 0.2-µm membrane (Millipore).

The *Fasciola* sp. E/S Ag was quantified through the bicinchoninic acid technique (BCA, Sigma Aldrich) and kept at − 80 °C until use.

Sensitization of the latex with mAb –IgG₃ 6G11E3 and pAb IgM against *Fasciola* sp. E/S Ag

The 0.8-µm polystyrene latex particles (Sigma, Aldrich) were suspended at 1% in glycine buffered saline (GBS) 0.054 M pH 8.2 and sensitized in 1:29 ratio with the pAb IgM and the mAb IgG₃ 6G11E3 previously obtained by our research group, “GYMOL”, which is directed against *Fasciola* sp. E/S Ag. These were homogenized at 8 °C during 12 h in gentle and constant agitation. Thereafter, the particles were washed three times per centrifugation at 1350×g for 3 min at 4 °C. The supernatant was discarded and replaced by the same buffer. Finally, the protein binding sites were blocked in the latex particles that remained free, using bovine albumin (Sigma Aldrich) at 0.1% (Veijalainen et al. 1986).

The test was standardized by using 0.8-µm latex particles at 1% of GBS 1× with a concentration of 444.5 µg/mL of the MAb IgG₃ 6G11E3 and 2025 µg/mL PAb IgM-type, in 1:29 ratio, with incubation at 8 °C during 12 h under agitation, running a cycle of three washes with GBS 1×, and subsequent centrifugation of the particles at 1350×g during 3 min at 4 °C, and a blocking step with serum bovine albumin at 0.1%. Prior to testing, the fecal matter was washed in PBS 0.02 M pH 7.2 in 1:9 ratio (feces: buffer). The mixture was homogenized and left to rest during 12 h at room temperature or centrifuged at 1350×g after 1 h of rest (without difference in the results). The test was run with the feces supernatant and the latex particles sensitized with ES/Ag at a 1:1 ratio, final volume of 100 µL.

Quantification of the coupling rate

To determine the percentage of Ac (IgG or IgM) coupled to the latex particles, the following formula was used: %A = ((IPC − FPC) × 100)/IPC, where IPC: initial protein concentration, FPC: free protein concentration in the supernatant after centrifugation of the latex reagent. Protein concentration was determined through spectrophotometry (reading at 562 nm) employing the BCA method (Cuello-Pérez et al. 2015). It was noted that the coupling rate was 85.77% for mAb IgG₃ 6G11E3 and 100% for pAb IgM.

Recognition of the latex particles sensitized against the *Fasciola* sp. E/S Ag

In a final volume of 100 µL, the sensitized particles were mixed in a 1:1(v/v) ratio with the Ac and *Fasciola* sp. E/S

Ag at a concentration of 1 mg/mL; on a dark background plate, these were gently agitated during 5 min in a Mazzini agitator at 180 rpm. PBS 1× was used as test negative control. The test was considered positive when it showed agglutination and negative if it maintained its initial aspect. Tests were run in duplicate and the readings were made through direct observation, under good illumination and according to their agglutination in a quantitative scale of crosses, with 1+: slight agglutination, 2+: medium agglutination, 3+: strong agglutination.

Determination of detectable concentration of the *Fasciola* sp. E/S Ag

To identify the minimum detectable concentration through the test, a serial 1:2 dilution was made of the *Fasciola* sp. E/S Ag in PBS 1×, starting from an initial concentration of 6.357–0.158 mg/mL. PBS 1× and sensitized latex were used as negative control.

***Fasciola* sp. E/S Ag concentration, detectable in bovine cattle stool infected experimentally**

To conduct minimum antigen detection in fecal samples, experimental inoculations were carried out of the E/S Ag at 6.357 mg/ml and serially diluted (1:2) to 0.158 mg/mL in different bovine fecal samples. After being infected experimentally, the cattle feces were prepared by using the protocol described by (Veijalainen et al. 1986). The specimens were diluted in PBS 1×, 1:10 ratio (feces : PBS) (weight/volume), homogenized through vortex agitation and left to settle during 12 h at 4 °C; thereafter, they were centrifuged at 1350×*g* during 5 min at 4 °C. The supernatant was collected to run the test against the latex particles sensitized with antibodies against E/S Ag at a 1:1(v/v) ratio.

Determination of crossed reaction against antigens of helminths and protozoa

The latex particles previously sensitized with pAb IgM and mAb IgG₃ 6G11E3 were exposed with antigens of eggs and cysts of helminths and protozoa, like: *Hymenolepis* sp., *Taenia* sp., *Balantidium coli*, *Blastocystis* sp., *Trichuris trichiura*, *Ancylostoma caninum*, *Toxocara* sp., *Cyclospora* sp., *Entamoeba coli*, *Strongyloides stercoralis*, *Entamoeba histolytica*, *Giardia* sp., and *Cryptosporidium* sp. *Fasciola* sp. ES/Ag was used as positive control and PBS 1× as negative control. The antigens were obtained through sonication of the eggs or cysts, during 30 s; each antigen was diluted in PBS 1× pH 7.2 and its concentration was adjusted to 1 mg/mL (w/v).

Evaluation of the latex particles sensitized against bovine cattle stool

Once the technique was standardized, 86 specimens of bovine cattle stool were evaluated, which had been previously evaluated through direct ELISA using MAb IgG₃ 6G11E3 to determine the sensitivity of the latex test. The fecal matter was prepared the same way as the previously described experimental inoculation process.

Information analysis

The information obtained was analyzed and plotted with Microsoft Excel®. The sensitivity and specificity percentages of the test were carried out with the Graphpad Prism® program V 6.04 (San Diego California, USA).

Results

Detection capacity of the test, using *Fasciola* sp. E/S Ag

With the protocol standardized in this study, it was possible to identify that the detection capacity of the latex particles sensitized with the antibodies was 1.589 mg/mL for the latex particles sensitized with mAb IgG₃ 6G11E3 and 0.158 mg/mL for the latex particles sensitized with pAb IgM (Table 1).

Detection capacity of the test in feces infected experimentally with *Fasciola* sp. E/S Ag

Upon using the standardized test in this study with bovine cattle fresh feces infected experimentally with *Fasciola* sp. E/S Ag, it was possible to determine that its detection capacity is of 3.178 mg/mL for the latex particles sensitized with mAb IgG₃ 6G11E3 and 1.589 mg/mL for the latex particles sensitized with pAb IgM (Table 1).

Latex crossed-reaction test against other parasites

The crossed-reaction test against protozoan and helminth antigens showed agglutination against *Giardia* sp., and *Cryptosporidium* sp. antigens.

Evaluation of the latex test against bovine cattle stool

Table 2 shows the number of positive and negative specimens in the latex technique for *Fasciola* sp. E/S Ag. Of the 86 specimens analyzed, 26 positive (30.23%) and 60

Table 1 Agglutination reaction of sensitized latex with Ab IgG and IgM with GBS 1× buffer and antigen (6.357–0.158 mg/mL) diluted in PBS 1×, and the same antibodies but diluted in fresh cattle stools with PBS 1×

Concentration of antigen mg/mL, Latex sensitized with Ab in GBS 1× buffer diluted in PBS	Antibodies versus antigens diluted in PBS		Antibodies versus antigens diluted in fresh cattle stools with PBS	
	mAb IgG3 6G11E3	pAb IgM	mAb IgG3 6G11E3	pAb IgM
6.357	++	+++	+++	+++
6.039	++	+++	++	+++
4.767	+	++	++	++
3.178	+	++	+	++
2.383	+	++	–	++
1.589	+	+	–	+
0.794	–	+	–	–
0.158	–	+	–	–
0	–	–	–	–

GBS Glycine buffer solution 0.054 M

Table 2 Contingency table of latex sensitized with mAb IgG₃ 6G11E3 versus ELISA technique

Diagnostic TESTS	ELISA technique		
	Positive	Negative	Total
Latex sensitized with mAb IgG ₃ 6G11E3			
Positive	26	0	26
Negative	7	53	60
Total	33	53	86

IC confidence intervals

Table 3 Contingency table of latex sensitized with pAb IgM versus ELISA technique

Diagnostic Tests	ELISA Technique		
	Positive	Negative	Total
Latex sensitized with pAb IgM			
Positive	32	0	32
Negative	1	53	54
Total	33	53	86

IC confidence intervals

negative (69.76%) were obtained with the latex sensitized with mAb IgG₃ 6G11E3.

With the latex particles sensitized with pAb IgM there were 32 positive (36.04%) and 54 negative (63.95%). Each of the results was compared with the reference assay of the ELISA technique (reference test) previously standardized in the laboratory, of the same 86 specimens in which 33 were positive (38.37%) and 53 were negative (61.62%) (Table 3).

Sensitivity of the IgG and IgM latex test with the ELISA test was of 78.78 and 96.96%, respectively; specificity was found at 100% for both tests and sensitivity was at 78.79% (IgG) and 96.97% (IgM) (Table 4).

These results show high agreement between the latex and ELISA tests for both the MAb IgG₃ 6G11E3 (kappa index: 0.82) and pAb IgM assays (kappa index 0.97); however, the kappa coefficient value was higher in the Latex test sensitized with pAb IgM than in the other one sensitized with mAb IgG₃ 6G11E3 (comparison for kappa values, $P = 0.0285$) (Table 4).

Discussion

Currently, the methods available to diagnose fasciolosis are optical microscopy and—less frequently—the determination of the presence of specific antibodies; nevertheless, several reports provide evidence that these types of diagnostics are often too late because, in the case of optical microscopy, at least 16 weeks must transpire for the parasite eggs to start to appear in feces. By that time, the adult worms are already installed in the bile conducts, generating processes of calcification, inflammation, and irreversible damage. In addition, neither of these two diagnostic methods reveals the parasite burden present in the infected individual (ruminants or humans), or if the infection is current or if it is an immunological trace with resolved infections, as in serology (Anuracpreeda et al. 2016; Farrell et al. 2015; Miranda-Ulloa et al. 2014). This makes it necessary to develop sensitive and effective alternative techniques through which it may be possible to have early diagnosis of the infection (Valero et al. 2009).

Table 4 Evaluation of Sensitivity, Specificity and agreement degree (kappa coefficient) of latex sensitized with mAb IgG₃ 6G11E3 and pAb IgM versus ELISA technique

Parameter evaluated	Evaluated tests	
	Latex sensitized with MAb IgG ₃ 6G11E3 No. or % (95% CI)	Latex test sensitized with PAb IgM No. or % (95% CI)
Prevalence	26.23 (19.94–40.52)	38.37 (25.51–49.23)
Sensitivity	78.79% (63.32–94.25)	96.97 (89.61–100)
Specificity	100.0% (99.06–100)	100.00% (99.06–100)
Concordance		
Kappa coefficient (Index)	0.82 (0.69–0.95)	0.97 (0.93–1.00)
Standard error	0.0639	0.0246

Comparison for kappa values, $P = 0.0285$

Based on the aforementioned, efforts have been made focusing on the search for sensitive and specific diagnostic alternatives, which can be performed during the pre-patent period of the infection. Examples include the Fascidig technique (Espino and Finlay 1990), the copro-ELISA MM3 (Valero et al. 2009) or detection through immunochromatography of the circulating cathepsin L1 protease (Anuracpreeda et al. 2016). Cathepsins are released during processes of invasion, migration, growth, and final establishment of the parasite in the bile conducts of the host and have been the principal molecules studied in the search for a new diagnostic method that manages to detect them before the appearance of the parasite's eggs in feces (Cancela et al. 2008; Muiño et al. 2011). Some of the copro-immunological methods developed to date show favorable detection results; however, it must be highlighted that these are promising results with high technical limitations, given that their execution requires lab equipment and technicians with skills to perform these types of tests (Darani et al. 2010; Mohammadi et al. 2013; Yu et al. 2011).

For *Fasciola* sp., no reports were found in the literature consulted on the development of rapid tests based on the latex agglutination reaction, although for other microorganisms (bacteria, protozoa) latex tests have proven highly useful with results > 95% of specificity and sensitivity (Cuello-Pérez et al. 2015; Darani et al. 2010; Miranda-Ulloa et al. 2014; Mohammadi et al. 2013; Yu et al. 2011).

The test results obtained indicate greater detection capacity when using latex beads, sensitized with mouse PAb IgM, due to its pentameric structure, which permits binding one-by-one the on the FAB sites and leaving other remaining sites available. This makes this type of molecule more viable for use in this type of test; however, it is limited by the difficulty to obtain it, given that each process of immunization and sacrifice of laboratory animals leads to different results. Nonetheless, different animal models can be used, like rabbits, sheep, or cows in which the serum

volumes are higher and, hence, would generate large production lots (Leenaars and Hendriksen 2005). With respect to the results obtained in Table 1, it can be stated that when working with feces supernatant, a high concentration of antigen is required to be detected. This may be due to the presence of proteases in the gastrointestinal tract, whether of endogenous or exogenous origin (bacteria or protozoa) (Mkaouar et al. 2016).

The latex test for both antibodies showed crossed reaction with *Giardia* sp. and *Cryptosporidium* sp. antigen. The possible reason for these reactions would be the use of an antigen that not only has specific proteins of the parasite, but also proteins common among parasites. Similar results were shown by Miranda-Ulloa et al. 2014, in the study on cysticercosis in Peru, where crossed reaction was found among *Giardia* sp. and *Fasciola* sp. antigens, through Dot Blot and latex agglutination tests. Another likely cause of crossed reaction may be due to the homology among the proteins of these parasites (Kindt et al. 2007; Miranda-Ulloa et al. 2014).

In spite of the crossed reactions shown by the latex tests, it is important to highlight that studies have demonstrated the existence of a very good correlation with similar specificity and sensitivity results among different techniques for the same diagnostic test. This is why these tests are considered highly promising to be used—especially—in rapid and economic screening processes, which can be conducted in the field and do not require sophisticated equipment or specialized technical personnel (Nagalingam et al. 2015; Senthilkumar et al. 2010; Uslu et al. 2016).

Conclusion

The latex test described, herein, provides a rapid and sensitive method to detect *Fasciola* sp. E/S Ag, for easy use in the field and without major technical preparation. In spite of showing crossed reaction with *Giardia* sp. and

Cryptosporidium sp. antigens, we may conclude that with the standardized methodology we can conduct rapid screening processes of early stages at bovine and human levels. The method can also be used in waters or vegetables to later continue with a more complex and differential clinical diagnosis process that manages to identify the etiological agent of the infection. It must be recognized that *Giardia* sp., and *Cryptosporidium* sp. have been implicated as causal agents of diarrhea in dairy calves and are common intestinal parasites of a wide range of vertebrates (McAllister et al. 2005).

Recommendations

We recommend conducting the initial immunization process with previously purified proteins, with molecular weight characteristics different from the common antigens present in *Giardia* sp., and *Cryptosporidium* sp.; thereby, allowing for the response generated to increase its percentage of specificity.

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Authors' contribution LPOÁ and EMI-B performed laboratory tests and wrote the first draft of the manuscript. DPR-R, CAR-S, LPS, and JCC generated the project's idea, coordinated the project, and were in charge of the revision and final draft of the manuscript.

Compliance with ethical standards

Conflict of interest The authors, herein, declare no conflict of interest.

Ethical approval During the process to obtain serum protein containing PAb IgM, the animal was maintained in good hygienic conditions and fed constantly; trained staff performed the inoculation process and sacrifice to minimize the risk of death or suffering during the procedure. This project used the least amount of reagents to avoid large quantities of chemical and biological wastes. All the biological material in the laboratory was treated with adequate biosecurity standards, thus, preventing contamination of the area. The project was fully carried out in the Biomedical Research Center, CIBM, at Universidad del Quindío, adhering to protocols and management under the responsibility of the researchers and the Molecular Immunology Group "GYMOL".

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