Veterinary Parasitology 190 (2012) 87–94

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

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

Differential diagnosis of equine cestodosis based on E/S and somatic *Anoplocephala perfoliata* and *Anoplocephala magna* antigens

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ARTICLE INFO

Article history:
Received 17 February 2012
Received in revised form 1 June 2012
Accepted 5 June 2012

Keywords:
Anoplocephala magna
A. perfoliata
Antigens
Immunodiagnosis

ABSTRACT

The tapeworm responsible for equine colic, *Anoplocephala perfoliata*, is considered the most common intestinal tapeworm of horses worldwide. However, there is evidence that *Anoplocephala magna* has a similar prevalence in North America and Spain, and possibly in other countries, highlighting the need for diagnostic methods capable of distinguishing between these two species. Currently, immunodiagnosis of *A. perfoliata* is based on the identification of the 12/13 kDa excretory/secretory (E/S) *A. perfoliata* immunoreactive antigen, which while apparently specific, has never been tested in sera from *A. magna*-positive horses. Accordingly, we evaluated the specificity of 12/13 kDa E/S *A. perfoliata* antigen for the first time by testing this crude antigen against *A. magna*-positive sera in Western blot. In addition, we characterized a somatic (Som) crude antigen of *A. perfoliata* and for the first time, the E/S and Som crude antigens of *A. magna*, evaluating their potential utility for the differential serodiagnosis of equine anoplocephalosis in sera from horses of known parasitic status. SDS-PAGE revealed major low MW bands at: 14 and 12 kDa for E/S and Som-*A. magna*; 14 and 11 kDa for E/S *A. perfoliata*; and 11 and 10 kDa for Som-*A. perfoliata*. Protein regions at 12–14 kDa (E/S *A. perfoliata*), 10–15 kDa (Som-*A. perfoliata*) and 10–12 kDa (Som-*A. magna*) were recognized by *Anoplocephala*-positive sera at the genus but not the species level. These findings demonstrate cross-reactivity of these unpurified antigenic components, precluding their use in differential diagnosis between *A. perfoliata* and *A. magna*. Although these results do not directly indicate cross reactivity at the purified 12/13 kDa component of the E/S *A. perfoliata* antigen, it is possible that current immunodiagnostic methods based on this component might not accurately differentiate between these two tapeworm species, suggesting erroneous diagnosis of *A. perfoliata* in areas where *A. magna* is present.

1. Introduction

Anoplocephala perfoliata (Goeze, 1782), a cestode of the Anoplocephalidae family in the order Cyclophyllidea, is a common intestinal tapeworm of horses worldwide (Gasser et al., 2005). This cestode has been increasingly associated with bowel irritation, intussusceptions and intestinal obstruction in horses (review by Gasser et al., 2005). Significantly, *A. perfoliata* increases the gravity of lesions and the risk of ileal impaction and spasmodic colic, particularly in horses chronically infected with many tapeworms (Proudman and Edwards, 1993; Proudman et al., 1998; Tinker et al., 1997; Rodríguez-Bertos et al., 1999; Traub-Dargatz et al., 2001). Accurate detection of this cestode is crucial for both epidemiological and clinical reasons. To date, several diagnostic strategies have been employed, including the detection of parasite eggs in faeces using centrifugation and flotation techniques (Beroza et al., 1986; Proudman and Edwards, 1992; Nilsson et al., 1995; Meana et al., 1998; Proudman and Trees, 1999; Rebhein

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http://dx.doi.org/10.1016/j.vetpar.2012.06.001

et al., 2010), the detection of parasite-specific antibodies in serum (Höglund et al., 1995; Proudman and Trees, 1996a,b), the detection of parasite antigens in faeces (Kania and Reinemeyer, 2005), and amplification of parasite-specific DNA in faeces by PCR (Traversa et al., 2008). The advantages and disadvantages of each approach regarding sensitivity and specificity have been reviewed (Gasser et al., 2005), and their efficacies evaluated (Traversa et al., 2008; Skotarek et al., 2010). To date, all available diagnostic methods have been designed assuming that *A. perfoliata* is the only epidemiologically significant tapeworm to parasitize domestic horses in most parts of the world. However, this assumption does not hold true in countries where *Anoplocephala magna* is at least as prevalent as *A. perfoliata*, such as USA (data compiled by Lichtenfels, 1975). In Spain, prior to 2002, *A. perfoliata* was the only major tapeworm considered in anti-helminthic control strategies for Spanish equines, due to the absence of *A. magna* and a very low prevalence of *Anoplocephaloides mamillana* (Cordero del Campillo et al., 1994). However, since the first detection of *A. magna* in abattoirs in North and Central Spain (Meana et al., 2002), this cestode has been frequently detected throughout the country (about 18% *A. magna* versus 24% *A. perfoliata* with 11% cases of mixed infection) (Meana et al., 2005).

A. magna (Abilgaard, 1789) is a cestode that parasitizes the fore-regions of the small intestine of equines. Little information is available regarding its possible pathogenic role and only a few reports of clinical disease have been associated with massive infections (Oliver et al., 1977; Faleiros et al., 2000). Given the limited pathogenicity of this tapeworm, the time consuming process of examining the entire small intestine and the failure of coprological tests to differentiate between *A. magna* and *A. perfoliata* eggs, *A. magna* infections generally pass unnoticed. Thus, a diagnostic method is required to discriminate between *A. perfoliata* and *A. magna* infections. Most of the currently available immunodiagnostic methods (Western blot analysis, serum-based ELISA, coproantigen ELISA) are based on the use of either E/S *A. perfoliata* crude antigen (Proudman and Trees, 1996a) or its purified 12–13 kDa immunoreactive component (Proudman and Trees, 1996b). The specificity of these antigens for the diagnosis of *A. perfoliata* is supported by the absence of cross-reactivity with protein antigens from *A. mamillana* and other helminths (Proudman and Trees, 1996a,b). However, to the best of our knowledge, this antigen has never been tested against *A. magna*-positive sera. In this study we tested *A. magna* sera against E/S *A. perfoliata* crude antigen for the first time in Western blots, evaluating the specificity of the 12/13 kDa component. In addition, we used SDS-PAGE to characterize the somatic crude antigen of *A. perfoliata* and, for the first time, the excretory/secretory and somatic crude antigens of *A. magna*, assessing their potential use in the differential serodiagnosis of equine anoplocephalosis by Western blot analysis. Our findings indicate the existence of low MW immunoreactive components of E/S *A. perfoliata*, Som-*A. perfoliata* and Som-*A. magna* antigens that are recognized by *Anoplocephala*-positive sera at the genus but not the species level. The data suggest that the tools currently available for immunodiagnosis may not accurately distinguish between these two species.

2. Materials and methods

2.1. Processing of individual horses

Full gastrointestinal tracts were obtained in multiple samplings between 2006 and 2010 from horses slaughtered at two abattoirs, one located in Segovia (Central Spain) and the other in León (Northern Spain). Digestive tracts were taken to the respective Veterinary Medicine faculties (University Complutense of Madrid or the University of León) within 1 h of slaughter and fully opened in the necropsy room, where the entire mucosal surface of each gastrointestinal tract ($n = 179$) was examined macroscopically for the presence of parasites. All tapeworms were removed, counted, classified as *A. perfoliata* or *A. magna* according to their location and morphology, and stored in phosphate buffered saline (PBS). A blood sample was taken when possible from the mesenteric veins of each gastrointestinal tract and the sera collected was stored at −20 °C.

2.2. Antigen preparation

E/S antigens for each *Anoplocephala* sp. were prepared as described by Proudman and Trees (1996a), with minor modifications. Live worms were washed twice for 5 min with a saline/glucose/antibiotic solution (0.9% NaCl, 2.5 mg ml^{-1} glucose, 200 U.I. ml^{-1} penicillin, 200 μg ml^{-1} streptomycin) at 37 °C. Groups of worms (40–60 *A. perfoliata* versus 10–20 *A. magna*) were placed in 100 ml of PBS (pH 7.2) and incubated at 37 °C for 5 h. Harvested incubation medium was centrifuged twice at 2000 × *g* for 20 min at 5 °C. The supernatants were then removed and, after the second spin, filtered successively through 1.8 μm, 0.8 μm and 0.22 μm syringe filters (ALBET®).

For somatic antigen preparation, groups of previously identified scolices and first proglottids of worms from each *Anoplocephala* sp. were selected (Proudman and Trees, 1996a), repeatedly washed in PBS and stored at −20 °C. After thawing, the material was collected in a small volume of PBS in a glass tissue grinder and homogenized on ice (Höglund et al., 1995). The homogenized material was sonicated 10 times (15 s per cycle with 15 s cooling intervals) at 20 kHz in an ultrasonic disintegrator (SONOPLUS HD 2070, LABOLAN, Spain). The sonicated material was then centrifuged for 30 min at 14,000 r.p.m. (Sahu et al., 2009) and the supernatant was collected and filtered successively through 1.8 μm, 0.8 μm and 0.22 μm syringe filters (ALBET®).

For both types of antigen, the protein was quantified by the method Bradford (1976) using the Protein Assay kit 2 (Bio-Rad, Hercules, USA), with bovine serum albumin as the standard. Samples were stored at −20 °C until use.

2.3. SDS-PAGE characterization

Several batches of each antigen were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at the Proteomics Facility