Dept. of Parasitology and Animal Diseases.
National Research Center, Dokki, Egypt.

DIAGNOSTIC VALUE OF SOME *PARASCARIS EQUORUM* ANTIGENS IN FOALS

(With One Table and 3 Figures)

By

H.A. SHALABY; M.M. ABDEL-AZIZ and S. ABDEL-SHAFY

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الأهمية التشخيصية لبعض مولدات الضد لديدان باراسكارس إيكورم في الأمهار

حاتم عبد الموجود شلبي ، محمد محمود عبد العزيز ، صبحي عبد الشافي حسن

في هذه الدراسة تم تقييم ثلاثة مولدات ضد لديدان باراسكارس إيكورم في تشخيص الإصابة في أمصال أمهار مصابة حقليا بالمرض أو بديدان أخرى باستخدام اختبار الإليزا. أظهر مولد الضد الإخراجي الإفرازي قدرة في تشخيص الإصابة بديدان الأسكارس، حتى مع وجود إصابة بديدان أخرى، تفوق بها على كلا من مولدي الضد الخام والبويضي. حيث أسفر التحليل الكهربي في البولي أكريلاميد جل عن وجود اختلاف تركيبي من حيث الوزن الجزيئي للبروتينيات المكونة لتلك مولدات الضد. وبإجراء الاختبار الإنزيمي المناعي لهذه البروتينيات (طريقة الطبع المناعي) باستخدام أمصال مصابة بديدان أسكارس، أظهرت معظم بروتينيات مولد الضد الإخراجي الإفرازي تفاعلات إيجابية مع هذه الأمصال في الوقت الذي كان فيه تفاعلات أقل مع بروتينيات مولدي الضد الخام والبويضي. هذا بدوره انعكس على حساسية مولد الضد الإخراجي الإفرازي لكي تكون ذات درجة عالية في الكشف عن الأجسام المضادة لديدان الأسكارس تفوق بها على كلا من مولدي الضد الخام والبويضي كما ظهر ذلك سابقا 酘 في اختبار الإليزا. ومن ثم امتدت هذه الدراسة لتعيين الحزم البروتينية المتخصصة لمولد الضد الإخراجي الإفرازي في الكشف عن الأجسام المضادة لديدان الأسكارس في أمصال أمهار مصابة بديدان أخرى باستخدام طريقة الطبع المناعي. حيث أوضح هذا الاختبار إنه من بين العديد من البروتينيات لمولد الضد الإخراجي الإفرازي التي أظهرت تفاعلات إيجابية مع أمصال الأمهار المصابة بديدان الأسكارس، كان البروتين ذو الوزن الجزيئي 28 كيلو دالتون متخصص في تشخيص الإصابة.

SUMMARY

Three *Parascaris equorum* antigens were evaluated in diagnosis of current infection with *P. equorum* in foals' sera using enzyme linked immunosorbant assay (ELISA) technique. The excretory-secretory antigen (ES) showed marked potency than the crude and egg antigens in

diagnosis of *P. equorum* infection, even that was mixed with other parasites. The electrophoretic make-up of the antigens, examined by sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE), revealed different patterns of separation. Most of the polypeptides of ES antigen were immunoreactive in enzyme linked immunotransfer blot (EITB) with *P. equorum* infected foals' sera and, in the meantime, more than that were recognized in crude and egg antigens. Those polypeptides might be reinforced the sensitivity of ES antigen to be higher than that of other tested antigens in detection of anti- *P. equorum* antibodies as observed in ELISA test. Those observations were extended by detection specific polypeptide band of *P. equorum* ES antigen using EITB. Where, from several polypeptides showed reactivity toward *P. equorum* infected foals' sera, the 28 KDa polypeptide was the only band that was not recognized with the other heterologous sera resuming a specific polypeptide band.

Key words: *Parascaris equorum, Foals, Antigen, ELISA, EITB.*

INTRODUCTION

Internal parasites are one of the greatest limiting factors to successful horse rising throughout the world. Most horses become infected and suffer a wide range of harmful effects ranging from impaired development and performance to death (Naviaux, 1985).

Roundworms were of particular concern in foals, because innate control of roundworm infection relied upon the acquisition of an immune response to the parasite. Infection with *Parascaris equorum* could develop in older horses, and not just those that were immuno-suppressed. Ascariasis had been diagnosed in horses 2 to 4 years of age. Nonetheless, the problem was most widespread in foals. Ascariasis was associated with poor weight gain, emaciation, respiratory disease (due to larval migration), and colic, which could be secondary to enteritis, impaction, or intussusception. Foals less than 6 months old were most susceptible to infection and were the source of the greatest egg production. Immunity was induced by exposure to infective eggs and antigens expressed by migrating larvae. Generally, after 6 months of age the foal's immune system limited migration of larval stages, although this could be associated with a more severe inflammatory response and clinical signs of respiratory disease (Murray, 2003).

Diagnosis of *P. eqourum* infection was achieved by identifying eggs in the faeces. However, faecal examination methods were time consuming and of low accuracy. In addition, by the time eggs were

produced, damage to the foal might be severe (Gasser *et al.*, 2004). Removal of this parasite from equids by use of parasiticides a few weeks before maturation, which required a minimum of about 10 weeks, lessened the possibility of pathologic effects (Lyons *et al.*, 2006). This would suggest that immunodiagnosis might be a suitable tool for assessment of this serious pathogenic parasite to apply early and accurate diagnosis on which treatment and control could be based. This approach focused on detection of antibodies in the serum of infected animals (Hillyer, 1993). Sensitivity and specificity of serological tests were mainly affected by the antigens used. Despite their satisfactory sensitivity, immunodiagnostic assays for parasitic infection were hampered by lack of specificity and this was attributable to the possession of common antigens by different helminthes (Abdel-Rahman and Abdel-Megeed, 2000). This phenomenon presented a problem in the serodiagnosis of infection and had led to various attempts to identify and isolate species specific antigens in order to enhance the specificity and sensitivity of diagnostic assays (Fagbemi *et al.*, 1997; Viyanant et al., 1997).

A little information is available about the immune response of horses to *P. equorum* infection. This encouraged us to evaluate serodiagnostic potential of three selected *P. equorum* antigens; egg, crude and excretory-secretory antigens, by enzyme linked immunosorbant assay (ELISA) and enzyme linked immunotransfer blot (EITB) techniques.

MATERIALS and METHODS

The selected animals:

The samples used in the present study were collected from naturally infected foals (5-12 months old) in some governmental horse farms. A total of 35 naturally infected faecal and jugular blood samples were selected from naturally infected foals. The samples were selected according to types of parasitic infections in faeces. Five samples from newly borne foals proved to be free from *P. equorum* infection were selected for the trial.

According to the faecal history of these samples, their serum samples were arranged in five groups as group I (G-I) was 11 foals shedding *P. equorum* eggs only. G-II was 7 foals shedding *Strongylus* spp. eggs only. G-III was 3 foals shedding *Trichostrogylus axei* eggs only. G-IV was 14 foals harboring mixed infection (*P. equorum*, *Trichostrongylus axei* and *Strongyloides westeri*). The last group was G-V contained 5 newly born foals with faeces free from parasitic infection.

Examination of the samples:

P. equorum and *S. westeri* were diagnosed using concentration floatation technique according to Soulsby (1982). Concerning *Strongylus* spp. and *Trichostrongylus axei*, cultivation of the collected faecal samples was done using the modified Baermann technique and the detected larvae were identified according to Robinson (1987).

Preparation of antigens:

***P. equorum* egg antigen:**

P. equorum egg antigen was prepared according to Abdel-Megeed and Abdel-Rahman (2003). *P. equorum* adults were recovered by expulsion of this parasite through the feces of naturally infected foals by administration of 100 mg/kg of piperazine. Mature females were dissected and the uteri and eggs removed. The eggs were washed 3 times with distilled water containing penicillin G (500 IU/ml) and gentamycine (50 mg/ml). Then, the collected eggs were sonicated with 0.01 M PBS, PH 7.4 for 10 minutes and subjected to high-speed centrifugation (10000 rpm) for one hour at 4ºC. The supernatant was separated as egg antigen after the protein content had been measured by the method of Lowry *et al.* (1951). The antigen was aliquoted and stored at – 70ºC until used.

***P. equorum* crude worm antigen:**

P. equorum crude worm antigen was prepared according to Shalaby (1998) from the anterior parts of fresh extracted adult *P. equorum* worms. They were washed repeatedly in 0.01 M PBS, PH 7.4. and homogenized at 6000 rpm for 20 minutes, and then subjected to high-speed centrifugation (10000 rpm) for one hour at 4ºC. The supernatant was separated as crude antigen after the protein content had been measured as above and stored at – 70ºC until used.

***P. equorum* excretory-secretory antigen (ES antigen):**

P. equorum ES antigen was prepared from fresh living adult worms according to Rivera-Marrero *et al.* (1988). After 3 hours incubation in 0.01 M PBS, PH 7.4 at 37ºC (40 worms/100 ml PBS), the supernatant was separated after centrifugation at 10000 rpm at 4ºC for one hour. The protein content was determined as above, and then the antigen was aliquoted and stored at – 70ºC until used.

ELISA technique:

ELISA test was done as described by Espino *et al.* (1987). Condition of the test and values of control serum were adjusted after checkerboard titration. The test was applied to determine the diagnostic value of different antigens versus the tested sera at 1:100 serum dilution.

Sensitivity of the ELISA and specificity of the tested antigens were evaluated according to Abdel-Rahman *et al.* (1998), where sensitivity is percentage of the positive sera among the total number of the original true positive samples and specificity is percentage of positive samples among the total number of tested samples at standard serum dilution. The cut-off value of the ELISA was calculated as the average plus three times the standard deviation of the OD 450 nm of sera from parasite-free foals at 1:100 dilution (Cornelissen *et al.*, 1992). It was 0.178, 0.358 and 0.147 for egg, crude and ES antigens, respectively.

SDS- PAGE and EITB techniques:

Protein fractions of each tested *P. equorum* antigens were demonstrated using 12% SDS-PAGE (100 µg/lane) according to Laemmli (1970) with the aid of prestained protein marker, broad range (New England Biolabs). The fractionated antigens were transferred onto nitrocellulose sheet for EITB technique according to Towbin *et al.* (1979) and used to recognize antigenically active polypeptides by *P. equorum* naturally infected foals' sera. Then, the nitrocellulose strips blotted with *P. equorum* ES antigen were tested in group of five as in Fig. 3.

- The first and second strips were allowed to react with *P. equorum* naturally infected only and mixed infected foals' sera, respectively.
- The third one was allowed to react with *Strongylus* spp. naturally infected foal's serum.
- The fourth one was allowed to react with *Trichostrongylus axei* naturally infected foal's serum.
- The fifth one was allowed to react with non-infected control foal's serum.

The molecular weight of specific and non-specific polypeptides was determined using broad molecular weight standard curve as described by the producer (New England Biolabs).

RESULTS

The three tested *P. equorum* antigens were evaluated in diagnosis of current infection with *P. equorum* in foals' sera using ELISA technique. The data in Table 1 revealed that *P. equorum* ES and crude antigens induced the same level of sensitivity (90.9%) and in the meantime, higher than that was induced by egg antigen (63.6%) in diagnosis of infection in foals harboring *P. equorum* only (G-I). On the other hand, the ES antigen appeared to be the most specific one in diagnosis of infection in foals harboring other parasites (G-II and G-III).

This level of specificity was 71.4 and 100% for ES antigen, while it was 57.1 and 66.7% for egg antigen and 42.9 and 33.3% for crude antigen, in animals of G-II and G-III, respectively. Where, ES antigen cross reacted by 28.6% with anti-*Strongylus* antibodies and did not react non-specifically with anti-*Trichostrongylus* antibodies. While, egg and crude antigens cross reacted by 42.9 and 57.1%, respectively, with anti-*Strongylus* antibodies and 33.3 and 66.7%, respectively, with anti-*Trichostrongylus* antibodies. The ES antigen induced higher sensitivity in diagnosis of *P. equorum* infection in foals harboring mixed infection (G-IV) in comparison with their crude and egg antigens. The diagnostic value of ES antigen was 85.7% followed by that of crude antigen (71.4). The lowest value in diagnosing mixed infection was recorded for the egg antigen (50.0%). None of all tested antigens showed reaction when testing versus the control negative foals' sera (G-V). In conclusion, the ES antigen showed marked potency than the crude and egg antigens in diagnosis of *P. equorum* infection, even that was mixed with other parasites.

Analysis by SDS-PAGE of *P. equorum* egg, crude and ES antigens revealed at least 4, 13 and 9 polypeptides in each antigen, respectively, as shown in Fig.1. These polypeptides molecular weight ranged from 6-215 KDa. EITB technique was performed to find out the antigenically active components in the three tested antigens that might be responsible for immunoreactivity with *P. equorum* infected foal sera among the several polypeptides of each antigen. The adopted EITB technique (Fig.2) revealed that one polypeptide of the egg antigen was identified at molecular weight of 46 KDa. Three polypeptides present in the crude antigen of molecular weights 80, 49 and 32 KDa were identified. While, six polypeptides at molecular weights of 181, 80, 61, 38, 33 and 28 KDa were recognized in the ES antigen. Thus, most of the polypeptides of ES antigen were immunoreactive and that reflected on its superiority than egg and crude antigens in diagnosis of *P. equorum* infection.

Those observations were extended by detection specific polypeptide band of *P. equorum* ES antigen, the EITB was performed against field collected serum samples from foals of known faecal history. As could be observed in Fig.3, six polypeptide bands of 181, 80, 61, 38, 33 and 28 KDa on nitrocellulose (NC) strips blotted with fractionated ES antigen were recognized with sera from *P. equorum* infected foals either with single infection or mixed with other types of parasites. On testing the same NC strips against the other heterologous

sera, five bands of 181, 80, 61, 38 and 33 KDa were recognized. Those bands reacted with sera from foals infected with *Strongylus* spp. resuming non-specific bands. The polypeptide bands at molecular weights of 181 and 80 KDa reacted with sera from foals infected with *Trichostrongylus axei*, while the 61 KDa band only reacted with non-infected control foals' sera. A characteristic molecular weight band of 28 KDa was recognized with all sera from foals naturally infected with *P. equorum*. This band was the most prominent band detected. All sera from foals naturally infected with *Strongylus* spp., *Trichostrongylus axei* and non-infected foals failed to recognize this definite band of ES antigen, therefore, it seem to be specific for *P. equorum*.

DISCUSSION

The accepted method for diagnosis of *P. equorum* infection has been microscopic examination of host faeces for eggs. Because positive diagnosis by this method requires mature egg-producing worms, extensive hepatopulmonary migration with resultant pathologic changes have occurred before such diagnosis is possible. Early diagnosis via immunological techniques would allow initiation of therapy before extensive larval migration and resultant pathologic changes. However, the ability to accurately diagnose infection immunologically has been hampered by lack of specificity because of immunological cross reactivity among phylogenetic related parasites (Nichol and Masterson, 1987). Hence, the present study focused on testing the diagnostic value of *P. equorum* egg, crude and ES antigens. To our knowledge, no studies have been done with these antigens in foals.

The present study concluded that the ES antigen showed marked potency than the crude and egg antigens in diagnosis of *P. equorum* infection. Although the ELISA with ES and crude antigens induced the same level of sensitivity (90.9%) in diagnosis of infection in foals harboring *P. equorum* only (G-I). It was preferred to use ES antigen because of higher yield in group of foals with mixed infection. Moreover, the OD 450 nm values of the control foals were lower when ES antigen was used. ELISA was previously adopted by Ghosh et al. (1998) to compare between larval somatic antigen and ES antigen of, a related nematode, *Toxocara vitulorum* in diagnosis of toxocariasis. By ELISA, they proved the potency of ES antigen than somatic one. The ES antigens of *T. vitulorum* infective larvae and adults were also utilized by Rajapaske *et al.* (1994) to determine the levels of antibody in sera and colostrums of buffalo cows naturally infected with *T. vitulorum* and sera of their calves using ELISA. Analysis of the specificity of the tested antigens revealed that the ES antigen was the most specific one, as the ELISA with crude and egg antigens scored two and one more foals, respectively, false positive than the ELISA with ES antigen in foals harboring other parasites. Possibly, the crude and egg antigens contained more antigens that cross reacted with antigens from other parasites. This was constant with Nichol and Masterson (1987) who demonstrated a high degree of cross reaction between surface antigens of *S. vulgaris* and *P. equorum* when probed in ELISA test with horse sera from single infection of both species. On the other hand, the evaluation of the specificity was limited to the extent that antibodies against the various

parasites themselves could not be measured. So we are not sure whether parasite-specific antibodies were present in the sera used to evaluate specificity. However, it is reasonable to assume that parasite specific antibodies were present, as all animals shed eggs at the time blood samples were collected.

Analysis by SDS-PAGE of *P. equorum* egg, crude and ES antigens revealed at least 4, 13 and 9 polypeptides in each antigen, respectively. Most of the polypeptides of ES antigen were immunoreactive in EITB with *P. equorum* infected foals' sera and, in the meantime, more than that were recognized in crude and egg antigens. Those polypeptides might be reinforced the sensitivity of ES antigen to be higher than that of other tested antigens in detection of anti- *P. equorum* antibodies as observed in ELISA test. In that sense, Santiago and Hillyer (1988) demonstrated that antigens in somatic antigen preparations that were predominantly recognized by sera from *F. hepatica* infected sheep differed from those predominantly recognized in products excreted by flukes.

Those observations were extended by detection specific polypeptide band of *P. equorum* ES antigen using EITB. Where, from several polypeptides showed reactivity toward *P. equorum* infected foals' sera, the 28 KDa polypeptide was the only band that was not recognized with the other heterologous sera resuming a specific polypeptide band. Indeed, some strongylid nematodes have been found to produce ES which contain species and stage-specific acetylcholinesterases (Ogilvie and Yeates, 1974). The production of such specific antigens could be the result of the physiological or immunological adaptations of these nematodes to the variety of environments which they encounter during their development (Wynne *et al.,* 1981). Further study on characterization of acetylcholinesterases of *P. equorum* is required to investigate weather the specific polypeptide band of molecular weight 28 KDa is one of them secreted by this nematode or not.

In conclusion, the ES antigen showed marked potency than the crude and egg antigens in diagnosis of *P. equorum* infection and its polypeptide band of 28 KDa appeared to offer the best specificity.

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Table 1: Value of *P. equorum* antigens in diagnosis of infection in foals using ELISA technique.

Animal group	Infection History (Coprological examination)	Animal no.	Tested antigens								
			Egg			Crude			ES		
			No. and % of positive samples	Sensitivity %	Specificity %	No. and % of positive samples	Sensitivity %	Specificity %	No. and % of positive samples	Sensitivity %	Specificity %
G-I	Foals shed *P. equorum* eggs only.	11	7 / 63.6	63.6	-	10 / 90.9	90.9	-	10 / 90.9	90.9	-
G-II	Foals shed *Strongylus* spp.eggs only.	7	3 / 42.9	-	57.1	4 / 57.1	-	42.9	2 / 28.6	-	71.4
G-III	Foals shed *Trichostrongylus axei* eggs only.	3	1 / 33.3	-	66.7	2 / 66.7	-	33.3	0 / 0.0	-	100
G-IV	*Foals harbored mixed infection	14	7 / 50.0	50.0	-	10 / 71.4	71.4	-	12 / 85.7	85.7	-
G-V	Non-infected control foals	5	0 / 0.0	-	100	0 / 0.0	-	100	0 / 0.0	-	100

* (*P. equorum, Trichostrongylus axei & Strongyloides westeri*)

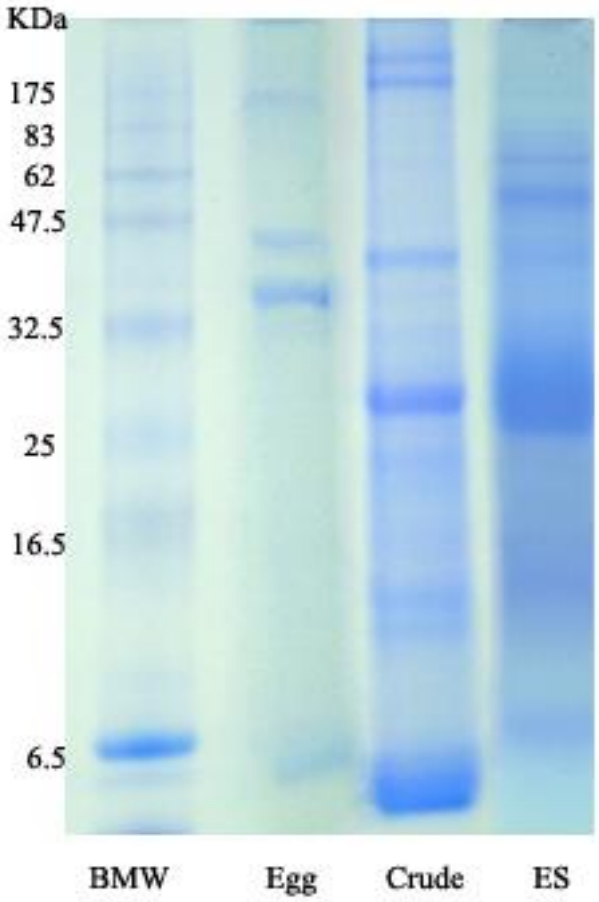

Fig. 1: SDS-PAGE of *P.equorum* egg, crude and ES antigens.
BMW. Broad range molecular weight marker.

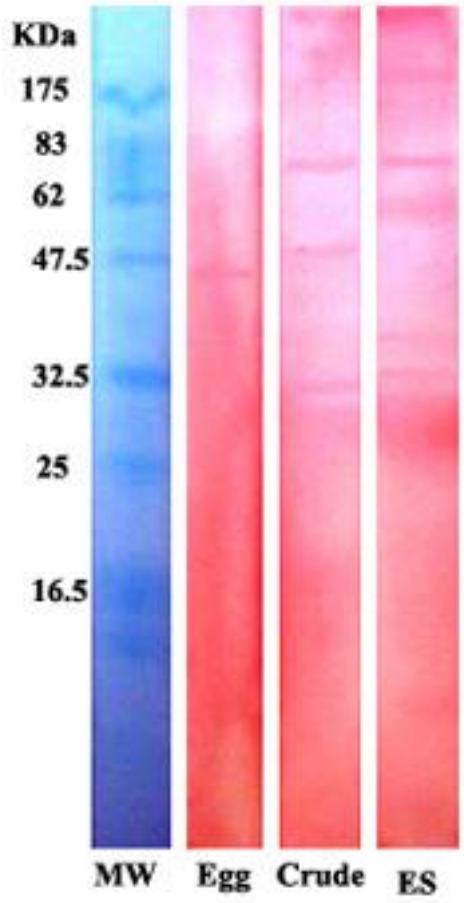

Fig. 2: Recognition of antigenically active polypeptides in the three tested *P. equorum* antigens by *P. equorum* naturally infected foals' sera using EITB technique.
MW. Molecular weight marker (prestained).

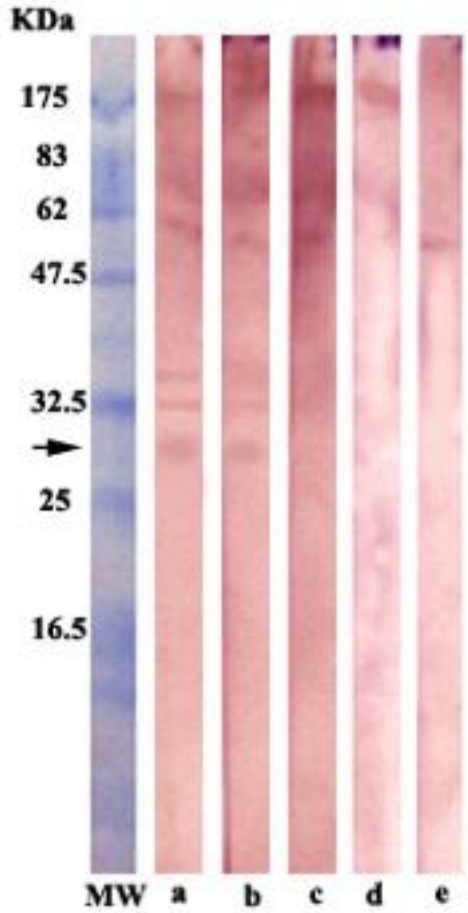

Fig. 3: Recognition of specific polypeptides of *P. equorum* ES antigen on NC strips using EITB technique.

Lane a. NC strip reacted with *P. equorum* naturally infected foal's serum.

Lane b. NC strip reacted with mixed infected foal's serum.

Lane c. NC strip reacted with *Strongylus* spp. naturally infected foal's serum.

Lane d. NC strip reacted with *Trichostrongylus axei* naturally infected foal's serum.

Lane e. NC strip reacted with non-infected control foal's serum.

MW. Molecular weight marker (prestained).

Arrow point at 28 KDa.