# VALUE OF POOLED GASTRO-INTESTINAL NEMATODE COPRO-ANTIGENS IN THE DIAGNOSIS OF INHIBITED LARVAE INFECTION IN EGYPTIAN SHEEP

**By**

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**Abstract**

Parasitic gastroenteritis (PGE) infecting sheep in acute or chronic forms causes severe economic losses. Infections by adult worms can be easily diagnosed and controlled, but infection by migrating and inhibited larvae are difficult to diagnose. Detection of anti-parasite antibodies in sera of infected animals needs special Ag for each species and presence of antibodies in sera does not reflect the actual infection. This study evaluated diagnostic efficacy of two pooled Copro Ag(s) prepared from feces of PGE infected sheep with larval5 species (*Haemonchus* spp., *Trichostrongylus* spp., *Nematodirus* spp., *Cooperia* spp. & *Ostertagia* spp.) in sheep. Diagnostic values of pooled Ag(s) were compared with two separate worm Ag(s) (*Haemonchus* spp. & *Trichostrongylus* spp.) using indirect and antigen capture ELISA. The results showed high sensitivity for pooled Copro and larval Ag(s) than individual parasitic Ag(s) in detecting Ab(s) in infected animals. Copro Ag showed high sensitivity (87.69%) than larval Ag (75.38%). For *Haemonchus* and *Trichostrongylus* Ag was 44.62% & 32.31% respectively. Pooled larval antigen showed high specificity (86.66%) than Copro Ag (78.33%), without cross reaction with non-infected controls. In migrating and inhibited larvae, pooled Ag showed 80% sensitivity in detecting PGE circulating antigen in animals without eggs in feces compared to 30% sensitivity using pooled Copro antigen by sandwich ELISA. Positive animals shed eggs in feces during follow up prior to parturition. Diagnosis of inhibited larvae circulating Ag(s) using pooled larval antigen was promising method in expecting time of auto-infection of sheep due to activation of arrested larvae within the bodies. This facilitated their therapeutic treatment at a critical time before the sudden re-appearance of infection.

**Keywords:** Parasitic gastroenteritis, sheep, Copro antigens, ELISA.

## Introduction

Parasitic Gastro-enteritis (PGE) is a widespread risky problem caused by Nematodes (order Strongyloidea) including many species as *Trichostrongylus* spp., *Ostertagia* spp., *Haemonchus* spp., *Cooperia* spp., and *Nematodirus* spp. infecting several hosts including ruminants (Roeber *et al,* 2013). The lambs parasites caused weight loss and reduction in productivity or even mortalities (Islam *et al,* 2017). *Haemonchus contortus* (blood-sucking parasite) caused significant blood loss (0.05ml/day), a decrease in erythrocytes, lymphocytes, hemoglobin, anemia and PCV (Gadahi *et al,* 2009). Under unfavorable conditions, usually at the end of the grazing season the third larval (L$_3$) stage during passage via stomach, loses its protective sheath, migrates in tissues transformed to a tissue or histotropic phase (hypobiotic), inhibited or arrested larvae. They undergo a period of arrested development prior to be L$_4$ and pre-adult stages. Tissue larvae resume activity and development in ewes in synchrony with the lambing season, causing a peri-parturient increase in fecal egg counts (FECs), contaminating pasture with L$_3$ stage that infects the animals while grazing (Roeber *et al*, 2013). Hypobiotic larvae infect animals causing clinical manifestations (Meradi *et al,* 2016). Gold standard diagnosis is microscopic examination (McHardy *et al,* 2014). But, it has several disadvantages as being time consuming, unreliable, unable to detect pre-patent infections and dependent on skilled technician (Mancera *et al,* 2016).

Due to morphological similarity of PGE eggs, fecal examination must followed by fecal culture to identify larvae, as there was no absolute correlation between worm burden and egg output (Demeler *et al,* 2010).

ELISA is an easily applicable serological test to evaluate a large number of samples, with accuracy depends on antigen degree of purity and specificity. It detects specific anti-parasite antibodies (AP-Ab) or circulating parasite antigens in sera or in feces (Sun *et al,* 2015). Parasite circulating antigens open a new field for accurate diagnosis of infection as circulating antigens is related to active infection (Johnson *et al,* 2004). The host usually contains different mixed infection, which deposit more or less similar eggs in feces and mixed forms of migrating larvae. Other than *Haemonchus* species, there are problems in preparation of pure specific Ag for each species indicated for serodiagnosis (El-Bahy *et al,* 2007). ELISA is a must for capturing antigens and reflect infection as being sensitive, easy to use, without expert microscopist.

The present study aimed to evaluate the diagnostic efficacy of two Copro-antigens prepared from feces of sheep heavily infected by different species of PGE identified by fecal culture. Two antigens, one was prepared from the whole PGE highly infected feces and second from pooled cultivated larvae. Diagnostic efficacy of both antigens in comparison with the other two PGE individual parasite Ag(s) was evaluated in detecting anti-PGE antibodies and PGE C-Antigens in sera of infected and suspected sheep using two types of ELISA. Inspecting animals that do not have PGE eggs in feces will be investigate to evaluate the efficacy of the used antigens in diagnosing the presence of inhibited larvae in these animals.

## Materials and Methods

Animals: A total of 30 naturally infected sheep (fecal examination) and 30 jugular blood samples were selected during two years investigation of 275 naturally infected sheep from Giza Governorate. They were divided into groups: GI: 20 sheep with low levels of PGE nematode infection (100-500eggs/gram feces or epg). GII: 20 sheep shedding 500-1000epg. GIII: 25 sheep shedding over 1000 epg. PGE nematode larvae in feces identified by fecal culture (Anamnart *et al,* 2015) GIV: 40 sheep (1-4 years old) parasite-free were selected from the previously examined animals (275). GIV was used to evaluate the antigens in diagnosing the parasitic stages of inhibited and/or tissue migrating larvae especially in ELISA positive animals. Appearance of PGE eggs in feces were followed up for enough periods after this.

For testing antigenic specificity, another four groups of sheep, each with single infection with another parasite detected by feces was divided into: GV: 15 sheep with *Fasciola* eggs, GVI: 10 sheep with *Paramphistomum* eggs, GVII: 15 sheep with *Moniezia* eggs and GVIII: 20 sheep with *Eimeria* oocysts. Fluke finder technique was used to diagnose *Fasciola* & *Paramphistomum* (Foreyt, 2005). Ten suckling lambs (2-3 weeks old) parasite-free were used as negative controls.

Fecal examination: Nematode eggs were counted for each sample by Mc-Master technique (Soulsby, 1986). Cultivated larvae were collected by modified Baermann technique (Foreyt, 2005), and identified (Wyk and Mayhew, 2013). Larvae were counted/ sheep &mean number/sheep in each group.

PGE Copro antigens of eggs and larvae: After cultivation of several heavily infected sheep (2000-4000/epg), five fecal samples with a high level of different PGE nematode larvae were selected. Fecal samples (5gm) with different eggs were mixed well with an equal amount of 0.01M PBS, PH 7.4 and processed for PGE eggs Copro antigen preparation (El-Bahy *et al*, 1992). After cultivation of pooled fecal samples from the previous five animals, a large amount of mixed larvae species (Tab. 1) was collected by Baermann technique. Larvae were washed using PBS and centrifugation (3000rpm/3min). After removal of supernatant, larvae were

mixed with an equal amount of PBS, homogenized at 6000rpm for 20 minutes in an ice bath (Ultra-Turrax Janke & Kunkel KG), and sonicated by sonifier cell disrupter. Soluble antigen was separated by centrifugation (20000rpm) for 1hr at $4^{o}$C. Supernatant was separated as a crude soluble antigen used as pooled larval Copro-antigen. Protein content was determined (Lowry *et al*, 1951), allocated in 1ml vial and stored at $-20^{o}$C until used.

Crude antigens of *Haemonchus, Trichostrongylus, Moniezia* scolices and *Paramphistomum* anterior sucker areas were separated from living worms collected from freshly slaughtered animals for antigen preparation. *Fasciola* excretory/secretory (ES) antigen was prepared (Khabisi *et al,* 2017)

Reference control serum: Sera of three sheep highly infected by PGE nematodes only and ten samples from parasite-free suckling lambs were used as reference positive and negative control.

Reference rabbit and rat hyper-immune serum (HIS): four 250gm rats and 4 one and half Kg rabbits were used as production of reference PGE hyper-immune sera vs. PGE Copro and larval Ag(s) (two rat and two rabbits for each Ag), Each animal was exposed to initial S/C injection followed by three consecutive intramuscularly injections of calculated dose of the Ag in an equal volumes of mineral oil during 4-5 weeks (Shalaby *et al*, 2014). Specific antibodies level in sera of immunized animals was evaluated 7-10 days after last poster dose before being slaughtered using indirect ELISA.

Indirect ELISA: Selected antigens, serum, and conjugate concentrations were determined after checkerboard titration. Positive ELISA equal to mean of negative control & 2 folds of standard deviation (Zimmerman *et al*, 1982). Sensitivity and specificity were evaluated after screening of selected sheep serum samples of low epg., high epg., and of other parasites (Attallah *et al,* 1997).

Sandwich (antigen capture) ELISA: PGE circulating antigens in suspected animal serum samples were detected using two anti-bodies sandwich ELISA. Tested conditions were adjusted after checkerboard titrations as before. High binding flat-bottom 96-well micrometer plates were coated with 100µl/well of 1:100 Rat HI serums (first reference serum) diluted in 0.1ml carbonate buffer (pH 9.6). Plates were incubated overnight at 4°C. After washing, two replicate of each tested suspected animal serum (1:50) were added and incubated for 3hrs at 37°C. After washing 100µl/well of the prepared Rabbit HI serum (second reference serum) (1:100 diluted in PBS) was added, then incubation at room temperature for 2hrs. After washing, 100µl of horse reddish peroxidase conjugate sheep anti-rabbit IgG, diluted 1:1000 within PBS was added to each well and incubated for 1hr. After washing, 100µl of O-phenylene diamine dihydrochloride substrate was added to each well. Reaction was left for 15 minutes at room temperature in dark and color change was measured (Liu *et al,* 2013).

Ethical approval: All steps and procedures were approved by the Institutional Animal Care & Use ethical Committee (CUIACUC), Cairo University. The study was done from August 2017 to July 2019 in Parasitology Laboratory, Faculty of Veterinary Medicine.

## Results

In the present study, two types of pooled PGE Copro-antigens (Copro Ag from feces of naturally heavily infected sheep and another pooled Ag prepared from different larvae obtained after cultivation of the previous infected fecal samples) as well as two individual parasite Ag(s) of *Haemonchus* and *Trichostrongylus spp.* were evaluated to diagnose apparent and in-apparent infection by these parasites in sheep. For these reasons, the investigated sheep were selected to be containing known different levels of infection as described in table1. Fecal examination of these selected sheep proved to be infected only by PGE nematodes after two successive investigations. They were allocated as GI contained 20 animals of low infection level with mean epg (300). GII contained 20 sheep of moderate infection level

with mean epg (650) and GIII with high infection level with mean epg (1850) among them five animals which were infected by more than 3500epg. Cultivation of fecal samples from all animals represented a different percentage of infection by five species of larvae as *Haemonchus* spp., *Trichostrongylus* spp., *Nematodirus* spp., *Cooperia* spp. and *Ostertagia* spp. A group of 40 animals (GIV) was selected which were free from PGE infection or larvae after cultivation of their fecal samples (Tab. 1)

Sensitivity of tested Ag(s) diagnosing anti-PGE Ab(s) in sera by indirect ELISA: Evaluating sensitivity of the previous four PGE Ag(s) in capturing the specific anti-PGE antibodies (PGE-Ab) in sera of sheep infected by different levels of parasites showed that Copro-egg Ag gave higher sensitivity (80-92%) with mean (87.69%) as compared with larva-Ag (70%-80%) with mean (75.38%). There was a direct relation between mean epg in tested group and level of detected Ab(s) in the sera. Screening of same sera vs. *Haemonchus* worm-Ag gave low mean sensitivity (44.62%) as compared with the other two antigens while *Trichostrongylus* crude Ag was able to diagnose PGE Ab(s) in sera of 25-40% of inspected animals with mean sensitivity up to 32.31%. No cross-reaction by using Ag(s) versus sera collected from 2-3 weeks old control lambs (Tab. 2).

Specificity of Ag(s) in diagnosis of anti-PGE Ab(s) in sera by ELISA: Testing speci

ficity of these Ag(s) in capturing Ab(s) of other parasites in groups of infected sheep showed that the antigens extracted from the pooled species of larvae gave higher mean specificity (86.66%) than that of Copro-Ag (78.33%). Both Ag(s) showed same specificity vs. anti-*Fasciola*, or *Paramphistomum* or *Moniezia* Ab(s) (86.66%-80.0%-86.66%). Main difference between them was low level of cross-reaction recorded using pooled larva-Ag vs. anti-*Eimeria* Ab(s) infected sheep. Also, separate *Haemonchus* and *Trichostrongylus* Ag(s) showed high mean specificity of 90% & 91.7% respectively (Tab. 3).

Determination of specific circulating PGE Ag(s)&Ab(s) in sera of sheep by ELISA and Sandwich ELISA: Both tested pooled Ag(s) were used to diagnose suspected PGE infection didn′t have eggs in feces. The 40 sheep without PGE eggs in feces were tested for circulating PGE Ab(s) using ELISA for PGE circulating Ag(s) in sera using antigen capture ELISA (sandwich) after preparation of required reference HIS by both pooled Ag. Sheep (17.5% &12.5%) showed anti-parasite Ab(s) versus both Copro-egg and larval antigens respectively. Re-examinationto detect PGE circulating Ag(s) in sera showed that 30% reacted positive versus the pooled Copro-Ag, but 32 sheep (80%) from them reacted positively vs. pooled larval Ag. Following up, 32 sheep had a true infection by inhibited larvae and shed PGE eggs 1-2 weeks before parturition in each case (Tab. 4).

Table 1: Level of PGE eggs and mean % of larval types in feces of sheep groups selected for evaluation.

| Group | No. of Animal | Total PGE eggs Detected (epg) | | *Haemonchus spp.* | *Trichostrongylus spp.* | *Nematodirus spp.* | *Cooperia spp.* | *Ostertagia spp* |
|---|---|---|---|---|---|---|---|---|
| | | Mean % of different larvae in 100 counted larvae | | | | | | |
| | | Low-high | Mean No. | Mean % | Mean % | Mean % | Mean % | Mean % |
| G I | 20 | 100-500 | 300 | 36 | 18 | 10 | 11.0 | 24 |
| G II | 20 | 500- 1000 | 650 | 29 | 24 | 12 | 8 | 27 |
| G III | 25 | Over 1000 | 1850 | 32 | 26 | 14 | 12 | 16 |
| G IV | 40 | -ve | -ve | - | - | - | - | - |

Table 2: Sensitivity of different Ag(s) in diagnosing anti-PGE Ab(s) in sera of PGE infected sheep using ELISA.

| Group No. And average PGE epg | Animal | ELISA results at (1:100 serum dilute) versus | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PGE Copro Ag | | PGE pooled larval Ag | | *Haemonchus spp.* crude Ag | | *Trichostrongylus spp.* Ag | |
| | No. | No +ve | % | No +ve | % | No +ve | % | No +ve | % |
| G I *100-500* | 20 | 16 | 80 | 14 | 70 | 8 | 40 | 5 | 25 |
| G II 500- 1000 | 20 | 18 | 90 | 16 | 80 | 10 | 50 | 8 | 40 |
| G III >1000 | 25 | 23 | 92 | 19 | 76 | 11 | 44 | 8 | 32 |
| Mean Sensitivity % | 65 | 57 | 87.69 | 49 | 75.38 | 29 | 44.62 | 21 | 32.31 |
| Non infected suckling lamb | 10 | 0 | - | 0 | - | 0 | - | 0 | - |

Table 3: Specificity of Ag(s) in diagnosing anti-PGE Ab(s) in sera using ELISA.

| Sera from sheep infected only by | Animal No. | ELISA results at (1:100 serum dilute) versus | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PGE Copro Ag | | PGE pooled larval Ag | | *Haemonchus spp* crude Ag | | *Trichostrongylus* spp. Ag. | |
| | | +ve | % | +ve | % | +ve | % | +ve | % |
| *Fasciola* | 15 | 2 | 86.66 | 2 | 86.66 | 2 | 86.66 | 2 | 86.66 |
| *Paramphistomum* | 10 | 2 | 80 | 2 | 80 | 1 | 90 | 1 | 90 |
| *Moniezia* | 15 | 2 | 86.66 | 2 | 86.66 | 1 | 93.33 | 1 | 93.33 |
| *Eimeria* | 20 | 7 | 65 | 2 | 90 | 2 | 90 | 1 | 95.0 |
| Mean specificity % | 60 | 13 | 78.33 | 8 | 86.66 | 6 | 90 | 5 | 91.7 |

Table 4: Specific circulating PGE Ag(s) &Ab(s) in sera of suspected sheep by ELISA &Sandwich ELISA.

| Technique used | No. of animals | No &% of +ve animals versus | | | |
|---|---|---|---|---|---|
| | | Copro Ag containing eggs | | Copro-larval Ag | |
| | | No. | % | No. | % |
| Indirect ELISA | 40 non-infected | 7 | 17.5 | 5 | 12.5 |
| | 10 suckling | 0 | | 0 | |
| Sandwich ELISA | 40 non-infected | 12 | 30 | 32* | 80 |
| | 10 suckling | 0 | | 0 | |

* All animals shed PGE eggs in feces prior to parturition in each case.

## Discussion

Diagnosis of PGE infection depends on microscopical determination of fecal egg counts. This method is hindered by several disadvantages as labor intensive, skilled microscopists and relatively unreliable (Mancera *et al*, 2016). The output of eggs did not usually correlated with the worm burden and eggs were not uniformly distributed in fecal samples. Coprological examination neither diagnose parasite during pre-patent period nor gave data about the arrested or inhibited larvae in animal body (Demeler *et al,* 2010).

Diagnosis of PGE infection by coprological examination was considered an easy method (Rojas *et al*, 2014), with a problem if suspecting animals didn't pass eggs in feces. Serodiagnosis by detection of specific parasite circulating antibodies using a technique like indirect ELISA proved an easy method, but needed some specific Ag (Sun *et al,* 2015). Circulating antibodies in serum cannot differentiate between old and recent infection where they can still for a certain period post-treatment (Mezo *et al*, 2004). PGE group usually infect the same animal by more than 3-5 species together and preparation of separate antigen from each species was considered difficult (El-Bahy *et al*, 2007). The present results showed high sensitivity for the pooled Copro and larval Ag(s) than the individual parasitic Ag(s) in

detection parasite Ab(s) in sera of infected animals. Copro Ag showed higher sensitivity (87.69%) than larval Ag (75.38%). While for *Haemonchus* Ag sensitivity was 44.62% and for *Trichostrongylus* Ag was 32.31 %. Meanwhile, no-cross reaction was recorded by using any of these Ag(s) versus non-infected control lambs. High superiority of Copro Ag than pooled larval Ag agreed with Rojas *et al.* (2014), Mancera *et al*. (2016) and Little *et al.* (2019) as Copro Ag contained excretory, secretary products and some eggs produced directly from worms in alimentary tracts. These Ab(s) in serum were directly related to the reaction of body versus adult worms as compared with the antigenic constituents of larvae hatched from eggs outside the body. The present lower sensitivity of individual Ag(s) of *Haemonchus* and *Trichostrongylus s*pp. can be att ributed to the recorded percentage of the larvae in fecal culture of examined animals. These two antigens showed a special tendency to diagnose Ab(s) related to t adult worm percentage in animals groups. Thus, the pooled mixed Copro Ag must exceed the efficacy of this single species Ag in tdetection of PGE specific Ab(s).

In the present study, both pooled Copro and larval Ag(s) have same specificity versus Ab(s) against *Fasciola*, *Paramphistomum* and *Moniezia*. Main difference was the

low level of cross-reaction by using pooled larval Ag versus anti-*Eimeria* Ab(s). Decreased specificity of Copro Ag to (78.33%), but that of larval Ag was (86.66%). Also, individual *Haemonchus* and *Trichostrongylus* Ag(s) showed high specificity; 90% & 91.7% respectively. Undoubtedly, PGE egg Ag(s) among Copro Ag caused this cross-reaction with *Eimeria*. The cross-reaction in animals infected by *Fasciola* or *Paramphistomum* or *Moniezia* versus both pooled Ag (s) of PGE was related to previous infection. Presence of eggs as compartments from Copro Ag was reported (El-Bahy *et al,* 1992). The high specificity of individual Ag(s) of *Haemonchus* and *Trichostrongylus* in comparison with that obtained by using pooled Copro Ag or larval Ag was acceptable to contain more specific characteristic epitopes (in single parasite) than in pooled Ag(s).

With the superiority of pooled Copro Ag in diagnosis of anti-PGE antibodies in animals' sera without eggs in feces, detection of circulating Ab(s) without value in suspecting future infection that did not passs eggs in feces. This may be attributed to the ability of the previous antibodies to remain for a period post-treatment and didn't relate to active infection (Mezo *et al*, 2004). Thus, antibody detecting ELISA was unable to suspect infection by arrested or inhibited larvae. The presence of inhibited larvae can become active and induce self-infection to animal causing severe problems especially with animal exposure to stress factors as prior to parturition in pregnant ewes. This agreed with Roeber *et al.* (2013) who found that hypobiosis, inhibition or arrested development causes a delay in parasite development in host, led to infection reappearance at a suitable time of the bad health condition of the infected hosts. They added that arrested larvae in intestinal tissues acted as a source of new infection even with the application of restricted control measures. Altif and Issa (1983) found that high re-infection in sheep by PGE coincided with lambing period related to release of inhibited larvae. But, presence of

circulating Ag(s) in sera and/or in feces was related to active infection by the target parasite (Attalah *et al*, 2013).

In the present study, circulating anti-PGE Ab(s) was recorded in 17.5% and 12.5% of sheep versus both Ag(s) respectively using indirect ELISA, diagnosis of the circulating Ag(s) in sera of sheep using S-ELISA revealed that 30% (12 animals) of 40 sheep reacted positively versus pooled Copro Ag, and 80% of which 32 reacted positively versus pooled larval Ag. The 32 animals had a true infection by inhibited larvae as they shed PGE eggs in feces at 1-2 weeks before parturition. But, superiority of Copro Ag to diagnose specific Ab(s) in sheep sera shedding PGE eggs, larval Ag gave high sensitivity to detect PGE circulating Ag(s) in those without eggs in feces. Ability of larval Ag to show infection by migrating or inhibited larvae was more important in preventing sudden infection in parasite-free animals.

## Conclusion

Copro-antigen allowed for rapid screening capacity, in surveillance with high sensitivity and specificity to diagnose specific parasite Ab(s) in sera but with low value in diagnosing inhibited larvae.

Pooled larval Ag after use of specific HIS in sheep is a new sensitive and specific method for PGE characteristic circulating Ag(s) in suspected sheep using S-ELISA, detected infection by inhibited larvae in sheep not passing eggs.

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