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Early Detection of *Haemonchus contortus* Infection in Sheep Using Three Different Faecal Occult Blood Tests

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Abstract: *Haemonchus contortus* is a blood-sucking parasite causing the presence of Faecal Occult Blood (FOB). The objective was to study three different FOB tests in order to have a new indicator of *H. contortus* infection in sheep that could be included in the genetic evaluation system as an alternative selection criterion to Faecal worm Egg Count (FEC). A total of 29 Corriedale lambs were artificially infected with 10 larvae of *H. contortus*. Stool samples were recorded for FEC and FOB tests (Hexagon, Hematest® and Multistix®), blood for Packed Cell Volume (PCV), haemoglobin, white and Red Blood Cell count (RBC) and FAMACHA© for scoring anaemia. At the end of the experiment lambs were slaughtered to worm burden count. Field validation was achieved in 309 Merino lambs under natural parasite challenge. FEC data was normalized through logarithmic transformation (lnFEC). Pearson correlation was estimated to examine the relationship between all traits. The three tests were able to detect the presence of FOB at day +11. FEC, PCV and RBC decreased to sub-normal values since day +18. FAMACHA© score 3 was considered to be indicative of anaemia. All correlations were significantly different from zero ($p<0.001$). The correlations between haematological parameters with lnFEC and FAMACHA© were high as well as between lnFEC with FAMACHA© and worm burden. Multistix® test was moderately correlated with lnFEC, haematological parameters and FAMACHA©. In field validation, most samples were negative to FOB tests and the correlations were lower than those calculated under artificial infection. In conclusion, FOB tests were able to detect haemonchosis earlier than FEC under high artificial parasite challenge. However, they were not able to detect FOB under natural mixed parasite challenge. FAMACHA© and PCV demonstrated to be good indicators of Haemonchosis having moderate to high correlations with FEC.

Key words: Faecal worm egg count, FAMACHA©, packed cell volume, parasite, correlations

INTRODUCTION

Gastrointestinal (GI) parasitism is one of the most important diseases in sheep production (Perry *et al*., 2002) causing important economical loss by a decrease in the production and by the costs of control measures. Faecal worm Egg Count (FEC) using the modified McMaster technique is currently the main method used by laboratories for the diagnosis of GI parasite infection and to determine the need for anthelmintic treatment. Furthermore, FEC has been considered as an indicator of resistance to nematodes and it is the most commonly selection criterion used when selecting animals for enhance GI parasite resistance (Bishop, 2011). However, FEC has a great disadvantage since it is unable to indicate a worm burden until egg output commences at 3-4 weeks after infection by which time the worms are well established and the host may already be suffering adverse effects (Kahn and Watson, 2001).

Haemonchus contortus (Barbers Pole worm) is the most predominant parasite genus present in Uruguay (Nari *et al*., 1977; Castells, 2009). It is a blood-feeding parasite of the abomasum of sheep and may cause high morbidity and mortality rates in a flock. *H. contortus* starts feeding at day 11 post-infection causing the presence of blood in the host's faeces. Since in *Haemonchus* sp. infections there is a strong correlation between blood loss and both worm burdens and worm egg production, it appeared that methods to detect haemoglobin or other blood products in sheep faeces would be a useful means of assessing infection levels (Le Jambre, 1995). As the presence of blood in faeces precedes the commencement

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of egg production (day 18 post-infection), FOB test might provide a leading or predictive indicator of the severity of GI parasite infection in comparison to the usual information provided by FEC (Colditz and Le Jambre, 2008). The aim of the present research was to study three different FOB tests in order to have a new indicator of haemonchosis in sheep that gives the possibility to be included in the genetic evaluation system as an alternative selection criterion to FEC.

MATERIALS AND METHODS

Animals and data collection: The first part of the study was carried out at La Magnolia an experimental station of the National Research Institute for Agricultural (INIA) located in the Northern part of Uruguay, between May and June (end of Autumn) of 2010. Records were obtained from 29, 6 months old castrated male lambs which were bought from a Corriedale commercial flock. Before the beginning of the experiment, animals were drenched orally with 2.5 mg kg^{-1} ZOLVIX® (Monepantel) in order to eliminate natural infections that might be presented. The 10 days later they were checked to verify that the treatment had been effective. It is well known that adult stages of liver flucke have bleeding suck activities (Soulsby, 1987) and can give rise to the appearance of blood in faeces. Similarly, coccidiosis produce diarrhoea streaked with blood in young sheep (Soulsby, 1987). Since these parasites can lead to positive FOB test results (Willis, 1921) and (Happich and Boray, 1969) techniques were assessed to discard the presence of these agents, respectively. Additionally, complete haemograms of each animal were made 1 week before beginning the experiment in order to discard concurrent infections that cause faecal blood loss (e.g., bacterial enteritis) that could interfere with the results of the present study. Animals were kept under housing system, not having access to natural pastures to avoid interferences with other parasitic genera. They were fed with a diet based on 70% corn and 30% sorghum, bales of alfalfa and water *ad libitum*. There was an adaptation period to the diet before the beginning of the experiment. During this period, animals were tested in order to discard false positive results by the interference of plant peroxidases with guaiac-based FOB tests. Lambs were artificially infected with 10.000 Larvae (L3) of *Haemonchus contortus* per os. Total L3 were administered in two doses, 5000 larvae on day +1 of the experiment and 5000 larvae 48 h later.

Animals were individually sampled for the following determinations: Stool samples for FEC and FOB tests: FEC were determined using a modified McMaster technique (Whitlock, 1948) where each egg observed represented 100 eggs per gram (epg) of faeces. The three FOB tests used for the detection of blood in faeces were Hexagon OBScreen®, Hematest® and Multistix®, all of them are based on the peroxidase-like activity of haemoglobin in catalyzing the oxidation by peroxide of a chromogen. The 2.5 mL of blood collected by jugular venepuncture in EDTA vacutainers to measure Packed Cell Volume (PCV), Haemoglobin (Hb), White Blood Cell count (WBC) and Red Blood Cell count (RBC). WBC and RBC were determined using a Neubauer chamber cell counting. FAMACHA© score on a 5-point scale for scoring anaemia on the basis of the colour of the conjunctiva membranes.

FEC, FOB tests, FAMACHA© and PCV were evaluated twice a week while Hb, WBC and RBC once a week during a month. At the end of the experiment (day +40), lambs were slaughtered and their worm burdens were recorded. However, if before day +40 animal welfare could be compromised (e.g., high FEC, FAMACHA© score 5, severe clinical signs), it was proceeded immediately to slaughter the animal. Final FEC samples were collected prior to euthanasia. This experiment was approved by the Honorary Committee of Animal Experimentation (CHEA) of the University of the Republic of Uruguay.

In 2011, field validation was achieved in the Fine Merino Nucleus, a flock belonging to Glencoe, another research station of INIA (Latitude 32°00'S and longitude 57°08'W). Records were obtained from 309 lambs, 177 males and 132 females, under natural mixed-species parasite challenge on pasture. Individuals were sampled for FEC, FOB tests, PCV and FAMACHA© score. Animals were measured on May 12 having on average 7.6 months old, corresponding this date with the FEC1 sampling according with the protocol to evaluate resistance to GI parasite in Uruguay. It consists in sampling 10-15 naturally infected post-weaning lambs randomly selected every 2 weeks, until FEC mean is 500 and no >10% of individuals have FEC values of zero. In that moment, all animals are sampled obtaining FEC1. They are dewormed immediately and the same process is repeated until is obtained FEC2. Faecal cultures of infective larvae were prepared in order to assess the species composition of nematode infection in that moment.

Statistical analysis: For FEC a transformation to $\log_e$(FEC+100) (lnFEC) was used to normalize distribution of data before analysis. lnFEC, FEC, PCV, Hb and RBC were analyzed by ANOVA for the effect of FAMACHA© and Multistix® score using the General Linear Models (GLM) Procedure of SAS (Statistical Analysis System,

Version 9.2, 2008). Pearson correlation coefficients were estimated through CORR procedure of SAS (Statistical Analysis System, Version 9.2, 2008) to examine the relationship between lnFEC, FEC, PCV, Hb, RBC, FAMACHA© and Multistix®. It was also calculated the correlation coefficient between lnFEC and FEC with total worm burden.

RESULTS AND DISCUSSION

Artificial challenge: The three tests were able to detect the presence of occult blood in the stool at 11 days post-infection. The mean values in days of each FOB test to become positive was: 12.2, 12.7 and 12.0 for Hexagon OBScreen®, Hematest® and Multistix®, respectively having the three tests a minimum value of 11 and a maximum of 18 days. The mean value in days for FEC was 18.9 having a minimum value of 14 and a maximum of 25 days. At day +11, Hematest® detected occult blood in 59% of the animals, Hexagon in 69% and Multistix® in 76% of them while FEC of all the animals was <100. At day +14, 93% were positive for Hexagon and Multistix® and 83% were positive to Hematest® and FEC mean continued to be <100. At day +18, 100% were positive for Hexagon and Multistix® and 97% were positive to Hematest® and FEC increased with a mean of 417 epg. In the present study, Hematest® was the test with the lowest sensitivity, since two animals that were positive at day +11 then were negative at day +14 and one lamb which was positive at day +14, it become negative at day +18. This result was not observed with the others tests, although there were changes in Multistix® scores in some animals. Three lambs with score 3 at day +11 then had score 2 at day +14. Additionally, four animals with score 3 at day +14, become two at day +18.

FAMACHA© score results in each measurement time point showed an increase of the higher scores over time. At day +5, animals had FAMACHA© scores of 1 or 2, while at day +25, 26 lambs (90%) had score ≥ 3. Moreover, FAMACHA© scores 1 and 2 were not statistically different ($p<0.05$) for FEC, PCV, Hb and RBC (Table 1). A FAMACHA© score of 3 was considered to be indicative of anaemia, corresponding to a mean FEC of 1758 (epg), a mean PCV value of 23% and a mean Hb value of 6.3 g dL^{-1}. For Multistix ®test, only score 1 was statistically different from scores 2, 3 and 4 for lnFEC and from scores 2 and 3 for FEC, PCV, Hb and RBC (Table 2). Thus, although it is a quantitative test, it performed as qualitative. Score 1 and 4 did not show statistically significant differences ($p>0.05$) for FEC, PCV, Hb and RBC because of the small amount of records with Multistix® score 4. However, it can be observed the great differences between least square means values for FEC and haematological parameters for score 1 and for score 4.

In Table 3 are represented the changes of blood parameters and FEC over time. Total WBC was constant amongst the measurement period and within the reference values (5000-6000 leucocytes mm^{-3}), demonstrating the absence of concomitant infections that could interfere with the experiment. Values of PCV, RBC and Hb decreased to subnormal values since day +18 post-infection being the mean values at this time 25%, 6.8 g dL^{-1} and 7.2×10^6 erythrocytes μL^{-1}, respectively. At day +25 mean values decreased to 16%, 4.5 g dL^{-1} and 4.5×10^6 erythrocytes μL^{-1}, respectively, showing the haematological effects of *H. contortus* infection. The FEC mean was <1,00 until day +14, increasing to 5266 epg at day +25.

All the correlations estimated were significantly different from zero ($p<0.001$) (Table 4). The correlations between lnFEC and the haematological parameters (PCV, Hb and RBC) were negative and high (<-0.70) and positive and high between lnFEC and FAMACHA© score. The correlations between FAMACHA© score and the haematological parameters were also negative and high

Table 1: Least square means (SE: Standard Error) from ANOVA analysis of Faecal worm Egg Count (FEC), FEC logarithmically transformed (lnFEC), Packed Cell Volume (PCV), Haemoglobin (Hb) and Red Blood Cell count (RBC) for the 5 scores of FAMACHA© System

FAMACHA©	lnFEC (Ln epg)	FEC (epg)	PCV (%)	Hb (g dL^{-1})	RBC (cells×10^6)
1	4.61 (0.17)^a	0 (300)^a	31.17 (1.08)^a	8.68 (0.37)^a	9.17 (0.41)^a
2	5.11 (0.13)^b	316 (223)^a	28.99 (0.66)^a	8.14 (0.22)^a	9.07 (0.25)^a
3	6.41 (0.14)^c	1758 (241)^b	23.24 (0.79)^b	6.33 (0.27)^b	6.89 (0.31)^b
4	7.32 (0.22)^d	3057 (395)^c	15.02 (1.21)^c	4.43 (0.39)^c	4.86 (0.44)^c
5	8.06 (0.35)^d	5083 (625)^d	09.40 (1.98)^d	2.50 (0.68)^d	3.00 (0.76)^d

*Different superscript letters by row indicate statistically significant differences ($p<0.05$)

Table 2: Least square means (SE: Standard Error) from ANOVA analysis of Faecal worm Egg Count (FEC), FEC logarithmically transformed (lnFEC), Packed Cell Volume (PCV), Haemoglobin (Hb) and Red Blood Cell count (RBC) for the four scores f Multistix® test

Multistix®	lnFEC (Ln epg)	FEC (epg)	PCV (%)	Hb (g dL^{-1})	RBC (cells×10^6)
1	4.61 (0.12)^a	0 (229)^a	29.74 (0.94)^a	8.03 (0.36)^a	8.93 (0.38)^a
2	5.95 (0.14)^b	1374 (272)^b	23.99 (0.94)^b	6.87 (0.32)^b	7.43 (0.34)^b
3	5.96 (0.15)^b	1359 (283)^b	21.47 (0.97)^b	5.99 (0.39)^b	6.64 (0.42)^b
4	6.92 (0.59)^b	1775 (1121)^{ab}	22.00 (3.83)^{ab}	5.90 (1.22)^{ab}	6.25 (1.33)^{ab}

*Different superscript letters by row indicate statistically significant differences ($p<0.05$)

(≤-0.70). These results showed that FAMACHA© System is a good indicator of *Haemonchus* sp. infection. The correlation between Multistix® with lnFEC, the haematological parameters and FAMACHA© score were moderate.

Some animals had to be slaughtered before the end of the experiment (10 lambs at day +28 and 7 at day +34) for the reasons explained in the present study. The correlation between worm burden and FEC was 0.67 (0.39-0.83) (95% confidence interval) and 0.68 (0.41-0.84) with lnFEC.

Field validation: Faecal cultures results (from FEC1 sampling) showed that *Haemonchus* sp. was the most prevalent parasite genus (68%) followed by *Trichostrongylus* sp. (20%) with other genera less abundant. The 89% of the records were negative to Multistix® (n = 258) and only a few were positive with a score of 2 (n = 32). All the samples that were negative to Multistix® were also negative to Hexagon test. From the 32 samples with score 2, 17 were positive and 15 negative to Hexagon test. Since, Hematest® was the test with the lowest sensitivity in the first part of the experiment and most samples were negative for the other two tests, it was decided not to perform this test. For Multistix® test, score 1 was statistically different ($p<0.05$) from score 2 for lnFEC and FEC, although it did not show statistically significant differences ($p>0.05$) for PCV.

From 306 PCV records, only 21 had sub-normal values (i.e., PCV<27%). From 309 FAMACHA© records, 122 had score 1, 141 score 2 and 46 score 3. Therefore, under natural infection, scores 4 and 5 were not observed. FAMACHA© scores 1, 2 and 3 were not statistically different for FEC and lnFEC ($p>0.05$). However, for PCV, least square means showed statistically significant differences ($p<0.01$) between the three FAMACHA© scores analyzed.

The correlation between FEC and lnFEC (0.94) was higher than the founded under artificial infection. Conversely, the rest of the correlations estimated were lower. The correlations between FEC and lnFEC with PCV were -0.37 and -0.41. The correlations between FAMACHA© with FEC, lnFEC and PCV were 0.19, 0.22 and -0.38, respectively. The Multistix® test had a low correlation with FEC and lnFEC (0.14 and 0.13, respectively) and the correlation with PCV did not differ statistically from zero ($p>0.05$).

The sex of the animals had a significant effect having the males higher FEC and lower PCV than the females. The values of the least squares means of FEC were 730 and 250 apg and the values of PCV were 29.8 and 33.9% for males and females, respectively.

Artificial challenge: The three tests were able to detect occult blood in the stool. The principal limitation of FOB test system is the unespecificity as bleedings into the gastrointestinal tract can be caused by several different pathologies. Additionally, the changes in Multistix® scores observed in some animals (seven lambs with score 3 that become 2 in the next measurement) might be explained for the sensitivity of this test and the subjectivity to interpret the results. Colditz and Le Jambre (2008) studied the capacity of Hemastix® test (Bayer, Australia) to determine the severity of *H. contortus* infection in sheep at pasture and they also found that the reagent sticks were able to detect blood in faeces. However, Wakid (2010) did not found a significant association between positive guaiac FOB test and infection with gastrointestinal parasites in humans.

A FAMACHA© score of 3 was considered to be indicative of anaemia which is in agreement with the studies of Kaplan *et al*. (2004) and Sotomaior *et al*. (2012). In addition, Kaplan *et al*. (2004) founded that FAMACHA© score of 3, 4 and 5 or 4 and 5 were considered anaemic and PCV values were considered anaemic if ≤19 or ≤15%, respectively.

For Multistix® test only score 1 was statistically different from scores 2 and 3 for PCV. In contrast with this

Table 3: Changes in Packed Cell Volume (PCV), Haemoglobin (Hb), Red Blood Cell count (RBC), White Cell count (WBC) and Faecal worm Egg Count (FEC) over time

Day post infection	PCV (%)	Hb (g dL^{-1})	RBC (erythrocytes mm^{-3})	WBC (leucocytes mm^{-3})	FEC
0	28.3	7.8	8.8×10^6	5919	0.0
5	-	-	-	-	0.0
8	30.4	-	-	-	0.0
11	34.2	8.9	9.7×10^6	5907	0.0
14	26.5	-	-	-	3.4
18	24.8	6.8	7.2×10^6	6128	417.0
20	16.5	-	-	-	748.0
25	15.7	4.5	5.0×10^6	5107	5266.0

Table 4: Pearson correlation coefficients* (95% confidence interval) between Faecal worm Egg Count (FEC), FEC logarithmically transformed (lnFEC), FAMACHA©, Multistix®, Packed Cell Volume (PCV), Haemoglobin (Hb) and Red Blood Cell count (RBC)

Correlation	FEC	PCV	Hb	RBC	FAMACHA©	Multistix®
lnFEC	0.84 (0.80, 0.87)	-0.71 (-0.63, -0.77)	-0.75 (-0.66, -082)	-0.75 (-0.66, 0.82)	0.63 (0.56, 0.700)	0.46 (0.35, 0.550)
FEC	-	-0.52 (-0.41, -0.61)	-0.63 (-0.51, -0.73)	-0.61 (-0.49, 0.72)	0.50 (0.40, 0.580)	0.25 (0.13, 0.370)
PCV	-	-	0.96 (0.94, 0 .97)	0.98 (0.97, 0.98)	-0.70 (-0.62,-0.760)	-0.37 (-0.24, -0.48)
Hb	-	-	-	0.94 (0.91, 0.96)	-0.73 (-0.63, -0.80)	-0.31 (-0.13, -0.46)
RBC	-	-	-	-	-0.70 (-0.59, -0.78)	-0.32 (-0.15, -0.47)
FAMACHA©	-	-	-	-	-	0.55 (0.46, 0.640)

All the correlations were statistically different from zero ($p<0.001$)

result (Colditz and Le Jambre, 2008) observed that haematocrit did not differ significantly for Hemastix® scores from 1-2.5 and then decreased significantly with subsequent increments in FOB score. They found that a decrease in haematocrit indicative of anaemia was evident at FOB score 3 and earlier. Moreover, Multistix® gave negative results in most animals when Hb values were under 9 g dL^{-1}. This observation can be explained by the sensitivity of the test itself since, blood loss would not be detected before day +11 post-infection. This is supported by the fact that after day +11 there were no more false negative results. The haematological parameters decreased to sub-normal values since day +18 post-infection. According to Colditz and Le Jambre (2008), in the early stages of *H. contortus* infection there is little impact of blood loss due to parasitism on erythrocyte parameters, therefore anaemia can be considered to be a lagging indicator of the severity of *H. contortus* infection.

The correlations estimated are in agreement with those reported by several researchers. Bisset *et al.* (2001) also observed high phenotypic correlations amongst lnFEC and FAMACHA© (0.59±0.04), lnFEC and PCV (-0.72±0.03) and FAMACHA© and PCV (-0.73±0.03). Vanimisetti *et al.* (2004) reported a correlation amongst lnFEC and PCV of -0.65. Kaplan *et al.* (2004) observed a similar correlation between FEC and PCV (-0.49) although, the correlations between FEC and FAMACHA© and PCV and FAMACHA© were lower than the estimated in the present study (0.21 and -0.52, respectively).

The correlation observed between worm burden and FEC was slightly lower than those reported by several authors. According to Pandey (1999), FEC during natural and experimental infections is correlated to worm burden, reporting a correlation of 0.75 in Indonesian sheep. The correlation between FEC and the number of Haemonchus worms was 0.86 in Corriedale lambs (Oliveira-Sequeira *et al*., 2000) and 0.83 in Merino sheep (Roberts and Swan, 1981). In Romney sheep, correlations between pre-slaughter FEC and total trichostrongyle burdens in lambs proved to be very high (0.91 and 0.85 for the 2 years studied) (Bisset *et al*., 1996). Conversely, Davies *et al.* (2005) founded that FEC was positively genetically correlated with worm burden (0.65±0.28) in 6 old months Scottish blackface lambs although, the phenotypic correlation was weaker (0.25±0.05).

Field validation: Larvae culture results confirmed the presence of *Haemonchus* sp. in agreement with those reported in Uruguay (Nari *et al*., 1977; Castells, 2009). Despite the high percentage of *Haemonchus* sp. due to probably the high biotic potential of this parasite, worm burden of this genus could be low, explaining the results that most samples were negative to Multistix® and Hexagon test.

According to the protocol followed in the validation sampling, probably most of the animals were in an early stage of parasitism and with low parasite burden (FEC mean value of 500 epg). These observation is supported by the PCV mean of 32.1% and the low percentage of samples with sub-normal values (6.8%) and that from 309 FAMACHA© records, 39.5% had score 1, 45.6% score 2 and 14.9% score 3. Therefore, animals could have been recently infected thus as it was discussed earlier in the early stages of *H. contortus* infection there is little impact of blood loss on erythrocyte parameters. On the other hand, it is being demonstrated that the protocol to evaluate the genetic resistance to GI parasite in Uruguay is not affecting animal welfare and health since most of PCV values and FAMACHA© scores were within the normal range.

The sex of the lambs had a significant effect which is in agreement with a great number of studies that reported that males are more susceptible to gastrointestinal parasites than females (Barger, 1993; Pandey, 1999).

CONCLUSION

FEC and the haematological parameters decreased to sub-normal values since day +18 post-infection. However, FOB tests were able to detect *Haemonchus contortus* infection earlier (since day +11) under artificial challenge and with high parasite burden. Nevertheless when animals were sampled under natural mixed-parasite infection these tests were unable to detect occult blood in faeces in most samples. It could be due to a low worm burden of this genus and/or because animals had been recently infected. This is supported by the observation that FEC mean was 500 epg, the 93.2% of PCV records had normal values and 85.1% of the individuals had FAMACHA© scores 1 or 2. FAMACHA© and PCV demonstrated to be good indicators of haemonchosis being moderately to highly correlated with FEC. Finally, FOB tests could be useful for early diagnosis of haemonchosis under high *Haemonchus* sp. parasite challenge. However, they are not recommended for routine diagnosis since most cases under natural challenge are composed by mixed-parasite species and *Haemonchus* sp. burden might not be high enough for these tests to detect occult blood in faeces. Additionally, it is important to take into account that this test system is very unspecific as bleeding into the gastrointestinal tract can be caused by several different pathologies.

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