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ORIGINAL PAPER

Passalurus ambiguus: new insights into copromicroscopic diagnosis and circadian rhythm of egg excretion

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Abstract The present paper reports a study on the in vivo diagnosis and egg excretion rhythm of the pinworm *Passalurus ambiguus* in domesticated rabbits. Three copromicroscopic techniques were compared: the cellophane tape test, the McMaster technique, and the FLOTAC technique. Out of the 51 New Zealand White rabbit does examined, 42 (82.3%) resulted positive when examined with the FLOTAC technique, 39 (76.5%) with the cellophane tape test, and 29 (56.9%) with the McMaster technique. The agreement between the FLOTAC technique and the cellophane tape test was almost perfect (greater than 0.8); only moderate were the agreements (0.4) between the FLOTAC and the McMaster techniques and between the McMaster technique and the cellophane tape test. The results showed that the FLOTAC technique can be used for the quali–quantitative coprological diagnosis of *P. ambiguus* in rabbits due to its great sensitivity, as already shown for parasites of other animal species. The circadian rhythm of egg excretion by *P. ambiguus* was studied utilizing 42 individually caged rabbit does; fecal samples were collected from each cage every 6 h, i.e., at 6:00–12:00 hours, 12:00–18:00 hours, 18:00–24:00 hours, and 24:00–6:00 hours, and were analyzed by the FLOTAC technique. A circadian rhythm of *P. ambiguus* egg excretion was found, with significant lower values at 6:00–12:00 hours. In conclusion, the present study showed that the FLOTAC technique is the best copromicroscopic method for assessing *P. ambiguus* prevalence and intensity in rabbits and that the afternoon and evening hours are the best times for fecal sampling to perform the pinworm diagnosis.

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Introduction

There is an increasing need to monitor infectious and parasitic disease of rabbits (*Oryctolagus cuniculus*) due to their zootechnical value (Shiibashi et al. 2006). Rabbit breeding is widespread in many parts of the world and, in particular, in some European countries, i.e., Spain, France, and Italy (Colin and Lebas 1996), where the farm typology is mainly characterized by intensive breeding techniques. This typology of breeding contributed to the decrease in parasites having indirect transmission and to the concurrent increase in parasites having direct transmission from host to host, such as *Eimeria* spp. and *Passalurus ambiguus*.

P. ambiguus Rudolphi, 1919 is a common oxyurid (pinworm) found in the caecum and colon of domestic, wild, and laboratory rabbits and hares (Owen 1972; Taffs 1976). The life cycle is direct. The sticky eggs are laid by the adult female worms, and larvae develop to the infective third stage within the egg, and infection occurs after the ingestion of these eggs, often during grooming or coprophagy (Boag et al. 2001). Among the helminths parasites of rabbits, *P. ambiguus* is the species most likely to be adapted to the intensive breeding farms, as its infective larvae do not leave the egg until the egg is ingested by the host (Grice and Prociv 1993).

P. ambiguus infections of rabbit are not generally thought to be very pathogenic, although very large numbers of pinworms were found in rabbits, where scouring was a problem (Ostler 1961), and fatal massive infections in young rabbits have been recorded (Owen 1972).

Table 1 Two-way contingency table of frequencies showing the agreement between the FLOTAC technique and the cellophane tape test for the diagnosis of *P. ambiguus* in rabbits

Diagnostic technique		Cellophane tape test		Total	K agreement
		Neg	Pos		
FLOTAC	Neg	9	0	9	0.821 (p=0.000)
	Pos	3	39	42	
Total		12	39	51	

The presence of *P. ambiguus* is reported in domesticated and wild rabbits throughout the world (Boag 1985, 1988; Quesada et al. 1987; Allan et al. 1999; Hobbs et al. 1999a,b; Pisanu et al. 2001; Foronda et al. 2003a,b; Epe et al. 2004); however, the studies regarding its diagnosis and biology are very scant. In particular, very little is known about the in vivo diagnosis of *P. ambiguus* in the intensive breeding rabbit farms; general methods of sedimentation, flotation, or direct smear for the identification of the characteristic eggs in the feces are usually mentioned (Taffs 1976).

The present paper reports a study on the copromicroscopic diagnosis, comparing three techniques, and circadian egg excretion rhythm of *P. ambiguus* in rabbits.

Materials and methods

Farm and animals

The study was conducted in an intensive breeding farm located in the municipality of Pozzuoli, province of Naples, situated in the Campania region of southern Italy.

Two hundred fifty New Zealand White does rabbits were bred on the farm. The parasitological analyses performed on the farm 2 weeks before the beginning of the study showed the presence of *P. ambiguus* and *Eimeria* spp. infections.

Experiment 1

The following three copromicroscopic techniques were compared for the in vivo diagnosis of *P. ambiguus*:

1. The cellophane tape test, considered as choice technique for the qualitative pinworm diagnosis in humans (Kucik et al. 2004)
2. The McMaster technique (MAFF 1986), the most universally used quali–quantitative technique for estimating the number of helminth eggs in animal feces (Cringoli et al. 2004)
3. The FLOTAC technique (Cringoli 2006), a novel multivalent quali–quantitative copromicroscopic technique in both veterinary and human field

Fifty-one New Zealand White rabbit does, individually caged, were used for the experiment.

The cellophane tape test was performed pressing the adhesive side of a piece of cellophane tape (2–3 cm) against the perianal region of each animal for a few seconds; then, the tape was stuck to a slide. Transportation time to the laboratory was 6 h. At laboratory, some drops of tap water were added to the air spaces between the tape and the slide, and finally, the slide was microscopically examined to detect the eggs of *P. ambiguus*.

To perform the McMaster and the FLOTAC techniques, 24-h fecal samples were collected by placing a wire netting under each cage of the 51 does. Transportation time to the laboratory was 6 h. Each sample was suspended in tap water at the dilution ratio of 1:10. The suspension was homogenized using a household mixer, filtered through a wire mesh (aperture=350 μm), and the debris discarded. Twelve milliliters of the filtered suspension was placed into a tube and centrifuged for 2 min at 1,500 rpm. The supernatant was poured off and discarded, leaving only a pellet in the tube. The tube was then filled with a sodium chloride flotation solution (specific gravity=1.200) to its previous 12-ml level and slowly agitated.

The resulting agitated suspension was then taken up by a pipette to load both the two chambers of the McMaster slide (Weber Scientific International, England) and the two chambers of the FLOTAC apparatus.

When the McMaster technique was utilized, the egg counts were started 10 min after loading the chambers, they were performed for both chambers (volume=1 ml), and a multiplication factor of 10 was used (Cringoli et al. 2004).

Table 2 Two-way contingency table of frequencies showing the agreement between the FLOTAC technique and the McMaster technique for the diagnosis of *P. ambiguus* in rabbits

Diagnostic technique		McMaster		Total	K agreement
		Neg	Pos		
FLOTAC	Neg	9	0	9	0.441 (p=0.001)
	Pos	13	29	42	
Total		22	29	51	

Table 3 Two-way contingency table of frequencies showing the agreement between the cellophane tape test and the McMaster technique for the diagnosis of *P. ambiguus* in rabbits

Diagnostic technique		McMaster		Total	K agreement
		Neg	Pos		
Cellophane tape test	Neg	10	2	12	0.408 (p=0.001)
	Pos	12	27	39	
Total		22	29	51	

When the FLOTAC technique was utilized, the egg counts were started after both centrifugation (5 min at 1,000 rpm) and translation, they were performed under both grids (volume=10 ml), and no multiplication factor was used (Cringoli 2006).

The agreement between the cellophane tape test, the McMaster technique, and the FLOTAC technique was evaluated by the kappa statistic (Thrusfield 1995).

Mean, standard deviation, and 25th, 50th, and 75th percentiles of eggs per gram (EPG) values were calculated for the McMaster and the FLOTAC techniques.

EPG values were not normally distributed, as detected by the analyses of the normality tests of Shapiro–Wilk ($p<0.05$) and the normal Q–Q plots. Thus, the values were transformed taking the natural logarithm of the EPG plus 1. One was added to each EPG value to circumvent the mathematically undefined logarithm of zero. Using these ln-transformed data, the paired t test was used to compare the EPG values produced by the McMaster technique and by the FLOTAC technique.

Experiment 2

The circadian rhythm of egg excretion by *P. ambiguus* was studied utilizing the 42 rabbit does that resulted positive with the FLOTAC technique in experiment 1 (see "Results").

Fecal samples were collected from the 42 rabbit does by placing a wire netting under each cage. They were collected every 6 h, i.e., at the following time intervals: 6:00–12:00 hours, 12:00–18:00 hours, 18:00–24:00 hours, and 24:00–6:00 hours. Once at laboratory, each sample was individually weighed, and the detection and count of *P. ambiguus* eggs was performed using the FLOTAC technique previously described.

Mean, standard deviation, and 25th, 50th, and 75th percentiles of fecal weights and EPG values were calculated for each sampling time.

The repeated measures analysis of variance was used to compare EPG values and the fecal weight at the different times, using ln-transformed data (because weight of feces and EPG values were not normally distributed). In addition, the reliability coefficient (Falconer 1989) was calculated as a measure of correlation between repeated fecal egg count at different hour intervals. The relationship between the fecal weight and the *P. ambiguus* EPG was also evaluated using Pearson correlation.

All the statistical analyses were performed using SPSS software (Version 13).

Results

Experiment 1

The results of experiment 1 are reported in the two-way contingency tables of frequencies (Tables 1, 2, and 3) showing the agreement between the three diagnostic techniques investigated.

Out of the 51 rabbit does examined, 42 (82.3%) resulted positive when examined with the FLOTAC technique, 39 (76.5%) with the cellophane tape test and 29 (56.9%) with the McMaster technique. According to the benchmarks proposed by Thrusfield (1995), the agreement between the FLOTAC technique and the cellophane tape test was almost perfect (0.821); whereas the agreements between the FLOTAC technique and the McMaster technique and between the McMaster technique and the cellophane tape test were only moderate (0.441 and 0.408, respectively).

Table 4 shows the EPG values (mean, standard deviation, percentiles) of *P. ambiguus* produced by the FLOTAC and the McMaster techniques. The mean EPG produced by the FLOTAC technique was significantly higher than that produced by the McMaster technique.

Table 4 EPG values (mean, standard error, and percentiles) of *P. ambiguus* detected by the FLOTAC and McMaster technique

Significant differences for equal letters ($p<0.05$)

Technique	Mean	Standard error	Percentiles		
			25th	50th	75th
FLOTAC	51.3[a]	7.4	14.0	36.0	72.2
McMaster	42.6[a]	7.5	0	20.0	60.0

Table 5 Fecal weight (mean, standard error, and percentiles) at the four different time intervals (6:00, 12:00, 18:00, and 24:00 hours)

Sampling time intervals (hours)	Mean	Standard error	Percentiles		
			25th	50th	75th
24:00–6:00	53.4[a, b]	4.1	35.6	53.0	76.4
6:00–12:00	23.9[a, c]	3.4	8.7	13.5	31.9
12:00–18:00	26.2[b, d]	4.4	6.5	19.3	40.0
18:00–24:00	55.4[c, d]	5.9	20.1	57.7	81.2

Significant differences for equal letters ($p<0.05$)

Experiment 2

Table 5 shows the weights (mean, standard deviation, percentiles) of feces excreted by the rabbits at the four different time intervals.

The amount of feces produced by the rabbits at 6:00–12:00 hours and 12:00–18:00 hours was significantly lower than that produced at 24:00–6:00 hours and 18:00–24:00 hours.

Table 6 shows the EPG values (mean, standard deviation, percentiles) of *P. ambiguus* found at the four different time intervals. A circadian rhythm of *P. ambiguus* egg excretion was found, with significantly lower values at the interval of 6:00–12:00 hours. In addition, the reliability analysis showed a coefficient of 0.244, also indicating a variation of *P. ambiguus* egg excretion during the day.

Pearson correlation did not show any relationship between fecal weight and *P. ambiguus* EPG ($p>0.05$).

Discussion

The findings of the present study showed new insights into the in vivo diagnosis and the circadian rhythm of egg excretion of *P. ambiguus*, a very common helminth of rabbits.

With respect to the in vivo diagnosis, according to the author's knowledge, only the McMaster technique had been previously used for the diagnosis of *P. ambiguus* in rabbits. Thus, the cellophane tape test and the FLOTAC technique were used for the first time for rabbits in the present study. The results showed that the cellophane tape test is a way to clinch the pinworm qualitative diagnosis in rabbits, as already reported for humans (Parija et al. 2001; Procop 2001). However, the highest number of positive results was obtained when the FLOTAC technique was used, whereas the cellophane tape test and the McMaster technique produced false negative results. The agreement between the FLOTAC technique and the cellophane tape test was almost perfect (greater than 0.8); only moderate were the agreements (0.4) between the FLOTAC and the McMaster techniques and between the McMaster technique and the cellophane tape test. Thus, the results reported in the present paper showed that the FLOTAC technique can be used for the quali–quantitative coprological diagnosis of *P. ambiguus* in rabbits due to its great sensitivity, as already shown for parasites of other animal species (Cringoli 2006). The FLOTAC technique permits the detection of helminth eggs and protozoa oocysts present in 1 g of feces. This technique does not require the extrapolation that is necessary when using other techniques, e.g., the standard or modified McMaster technique.

The prevalence and the intensity of helminths may be underestimated using the McMaster technique because a limited amount of feces is typically analyzed with this method (Mes 2003). Using the FLOTAC technique, a larger amount of fecal suspension is examined, namely, 10 ml, and so the sensitivity is greater; thus, this novel technique is less likely to give false negative results. In addition, because the FLOTAC technique is multivalent, it can be used for the simultaneous diagnosis of different helminths and protozoa of rabbits, which eliminate their propagules (eggs and oocysts) in the feces. Thus, it is the candidate for the standard fecal egg count technique in the intensive breeding rabbit farms.

With respect to the circadian rhythm of feces excretion, in according with our results, Jilge and Stahle (1984) also reported the minimum hard feces excretion in rabbits during the hours of light. Regarding the circadian rhythm of *P. ambiguus* egg excretion, the highest values were

Table 6 EPG values (mean, standard error, and percentiles) of *P. ambiguus* detected at the four different time intervals (6:00, 12:00, 18:00, and 24:00 hours)

Sampling time intervals (hours)	Mean	Standard error	Percentiles		
			25th	50th	75th
24:00–6:00	24.7[a]	4.4	4.0	12.0	33.5
6:00–12:00	14.6[a, b, c, d]	4.8	2.0	5.0	15.0
12:00–18:00	39.5[b]	14.3	2.0	10.0	46.0
18:00–24:00	22.9[d]	4.1	2.0	12.0	39.5

* Significant differences for equal letters ($P<0.05$).

recorded in the afternoon and night hours of the day. A similar circadian rhythm has been already shown for other pinworms, i.e., *Enterobius vermicularis* in humans (Felt and White 2005) and *Skrjabinema ovis* in sheep and goats (Sapozhnikov 1969). In contrast, Felt and White (2005) did not find any significant difference at specific times of the day in the detection of the pinworm *Trypanoxyuris microon* in owl monkeys (*Aotus nancymae*) using the cellophane tape test.

In conclusion, the present study showed that the FLOTAC technique is the best copromicroscopic method for assessing *P. ambiguus* prevalence and intensity in rabbits and that the afternoon and evening hours are the best times for fecal sampling to perform the pinworm diagnosis. Gathering of this kind of information is recommended as a previous step for any host–parasite study (Villanua et al. 2006).

Future studies on biology, epidemiology, pathogenesis, and therapeutic would prove useful in understanding factors that allow *P. ambiguus* to be a highly successful inhabitant of rabbits in the intensive breeding farms and will ultimately lead to the identification of future control and therapeutic strategies to eradicate it.

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