

**Diagnosis and monitoring of anthelmintic resistant gastro-intestinal  
nematodes of UK cattle: Development of a qPCR on L1 larvae of *O.*  
*ostertagi* and *C. oncophora*.**

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### **Author's Declaration**

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED: ..... DATE: .....

(Signature of student)

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# **Diagnosis and monitoring of anthelmintic resistant gastro-intestinal nematodes of UK cattle: Development of a qPCR on L1 larvae of *O. ostertagi* and *C. oncophora*.**

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## **Abstract**

Parasitic nematodes negatively impact on animal welfare and cost the cattle industry millions of pounds each year in lost production and treatment costs. The emergence of anthelmintic resistant nematodes will only increase this problem as infections will be more difficult to control. The aim of this study was to develop a better understanding of what farmers do to control parasitic nematodes on UK cattle farms, and evaluate the use of faecal egg counts (FEC) in conjunction with quantitative real-time PCR (qPCR) to assess the level and nature of an infection. Surveys were conducted of cattle farmers in South West England, regarding their cattle management practices. This revealed a number of farmers are still managing their land and animals in a way known to promote the development of anthelmintic resistance (AR) and increase the risk of clinical *O. ostertagi* and *C. oncophora* infections, including the use of anthelmintics in an unsustainable manner. A qPCR was developed to quantify the amount of *Cooperia* and *Ostertagia* in a mixed sample of L1 (stage 1) larvae, indicating the proportion of each species present. FEC and qPCR were performed on faecal samples from 16 farms, to measure the level of the infection (eggs per gram of faeces) and the ratio of *Ostertagia* to *Cooperia*. No link was found between the level of infection and ratio of species present. By using FEC and qPCR together, infections can be more accurately monitored at both herd and individual level. Response to anthelmintic treatment can also be assessed and used to indicate the development or presence of AR in nematodes. More work needs to be undertaken to educate farmers about better land and animal management, as this will reduce dependence on anthelmintics and delay the development of AR.

**Keywords:** Gastro-intestinal nematodes, Anthelmintic resistance, *Ostertagia ostertagi*, *Cooperia oncophora*, Quantitative Real-time PCR, Faecal egg counts.

## 1. Introduction

Gastro-intestinal (GI) nematodes are a common cause of reduced health and performance in young cattle, costing the cattle industry millions of pounds each year in the UK alone (Coles, 2001). Young animals in their first grazing season are particularly susceptible to high parasite burdens and clinical disease, leading to weight loss and reduced growth that can persist into later life (Mason & McKay, 2006; Ploeger *et al.*, 1996). Anthelmintics are the largest sector of the cattle pharmaceutical industry and have become an integral part of nematode control; the anthelmintics most commonly used in cattle are benzimidazoles, levamisole and ivermectin which may be delivered in injectable, oral or pour-on formulations. Farmers are accustomed to being able to prevent clinical disease with anthelmintics alone but the development of anthelmintic resistance (AR) in parasitic nematodes, causing them to not respond to anthelmintic treatment, is an increasing problem that threatens the sustainability of intensive farming practices (McKellar & Jackson, 2004).

There is a lack of recent research in how farmers prevent and control nematode infections in their cattle. Farmers in South West England were surveyed to gain an insight into nematode control programmes in current use and the extent to which cattle management practices that have been shown to predispose cattle to nematode infections are still being used. Up to date information about management practices related to the development of high worm burdens and AR will improve the quality of advice given to farmers regarding parasite control. Faecal egg counts were used to identify infected herds and estimate the numbers of nematodes present. A quantitative real-time PCR (qPCR) was developed based upon previous studies by Höglund *et al.* (2013) and Areskog *et al.* (2014), to detect and quantify the ratio of infection by *O. ostertagi* and *C. oncophora*, the two most common parasitic nematodes affecting cattle in temperate climates. Compared to traditional methods of characterising an infection, such as microscopy or controlled slaughter trials, qPCR is rapid, reliable and far more sensitive as it measures the amount of DNA present, rather than visually identifying morphological differences between species (Höglund *et al.*, 2013).

By using a combination of diagnostic methods, more descriptive information about the nature of an infection will be gained than by using each technique in isolation, therefore providing more accurate monitoring of how the infection responds to anthelmintic treatment or host and environmental factors, such as ageing, reproductive events and time of year. It is hoped that by monitoring and characterising the nature of GI nematode infections, AR can be detected whilst still at a low level in the parasite population, allowing actions to be taken to reverse or limit the spread of AR parasites (Kaplan, 2004).

## **2. Materials and methods**

### ***Surveys and Sample Collection***

Cattle farming clients of the Langford Vets Farm Animal Practice were contacted by telephone and asked if they were willing to receive a survey and faecal sample kit through the post. The survey asked the farmers about the animals they keep, their anthelmintic use and farm management practices relevant to nematode control. Faecal sample kits contained eight plastic pots to fill with freshly passed faecal samples from eight first grazing season (FGS) animals, to create a composite sample representative of FGS cattle on the farm. Participants were requested to fill the pots completely to produce anaerobic conditions and return the samples, via a freepost envelope included with the pots, as soon as possible after collection to prevent the eggs from hatching before they could be counted and extracted (McArthur *et al.*, 2011).

### ***Faecal Egg Counts***

Samples arrived throughout June and July, when cattle are typically at pasture. Faecal egg counts (FECs) were performed for each sample received on the day of arrival, using mini-FLOTAC, a flotation method described by Barda *et al.* (2013). A filtered suspension of 5g faeces in 45g saturated NaCl solution was pipetted into each of the two 1ml chambers in the mini-FLOTAC device and left for 10 minutes to allow the eggs to float upwards before being translated to where they could be counted under a microscope without interference by most of the faecal matter. When both chambers in the device are counted, each egg represents 5 eggs per gram (epg) of faeces.

### ***Nematode Egg Extraction***

Where nematode eggs were found in a sample they were extracted as per Coles *et al.* (1992). The composite sample was made into a suspension with water and filtered through a 150 micron sieve. The filtered suspension was centrifuged for three minutes at 1500rpm to produce a sediment containing the nematode eggs; any supernatant was discarded. The sediment was mixed with saturated NaCl solution and centrifuge tubes were filled with this solution, until a meniscus formed above the tube. A cover slip was placed on top before centrifuging at 1000rpm for three minutes to cause the eggs to float upwards and adhere to the cover slip. The eggs on the cover slip were rinsed off into a small centrifuge tubes with tap water and centrifuged at 1500rpm for 3 minutes to form a pellet of eggs in the bottom of the tube. The supernatant was discarded and the eggs re-suspended in fresh water before centrifuging again to wash away any remaining salt as this would inhibit hatching of the eggs. The eggs were washed with tap water a further three times this way before being transferred to a petri dish, re-suspended in water and incubated at 23°C overnight to hatch into L1 larvae. The L1s were pipetted into Eppendorf tubes to produce concentrated samples of approximately 100 larvae with minimal contamination and stored at -80°C in approximately 0.5ml of water until required.

### ***Quantitative PCR (qPCR)***

The frozen samples of larvae were thawed prior to genomic DNA extraction for the qPCR; 100µl of the sample was added to 180µl of ATL lysis buffer, 20µl of proteinase K and 100µl of dH<sub>2</sub>O and heated for 2 hours at 50°C. The standard Qiagen Tissue extraction protocol was then followed before eluting the DNA to a 200µl volume to produce the DNA template (Qiagen, 2006).

Each well used in the PCR plate contained 25µl of the reaction mixture; 12.5µl of GoTaq enzyme, 9µl of dH<sub>2</sub>O, 1µl each of the forward (F) and reverse (R) primers (1:10 dilution), 0.5µl of SyBr Green fluorescent probe, to allow detection of the PCR product (1:1000 dilution) and 1µl of the DNA template. The amount of DNA in each sample was unknown, so in addition to the 1µl DNA trial, tests using 5µl DNA were also run to account for samples with a lower DNA load. The 5µl DNA template wells were prepared the same way as the 1µl, except only 5µl of dH<sub>2</sub>O was needed to create the required volume. The qPCR was run on larvae of unknown ratios (presumed to be

mixed infections) from each of the 15 faecal samples containing nematode eggs, controls containing known ratios of *O. ostertagi* and *C. oncophora* and negative controls. Each sample was run with 4 combinations of primer and DNA template volume; *Ostertagia* primers/1µl DNA, *Ostertagia* primers/5µl DNA, *Cooperia* primers/1µl DNA and *Cooperia* primers/5µl DNA. The primer sequences used were *Cooperia* ITS2 F: 5' TAA TGG CAT TTG TCT ACA TCT 3', *Cooperia* ITS2 R: 5' ATG ATA ACG AAT ACT ACT ATC T 3', *Ostertagia* ITS2 F: 5' GTC GAA TGG TAT TTA TTA CT 3' and *Ostertagia* ITS2 R: 5' TTA GTT TCT TTT CCT CCG CT 3'. Samples were run in a Stratagene Mx3005P qPCR machine. Cycling conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 58°C for 60 seconds and 72°C for 60 seconds, plus a final cycle of 95°C for 1 minute, 55°C for 30 seconds and 95°C for 30 seconds to produce a dissociation curve.

The proportion of DNA contributed by each species to the DNA template can be determined by the threshold cycle (Ct) value, which is the number of cycles at which the fluorescent signal (from the SyBr Green) from the PCR product crosses a certain threshold, set at 5000dR in the current study (Wong & Medrano, 2005). The more DNA present in the sample to begin with the lower the Ct value is expected to be. Ct values close to the total number of cycles (40) indicate little to no target DNA in the sample. The dissociation curves show reaction specificity; if a number of PCR products are being made then multiple peaks will be observed on the dissociation curve analysis due to the different melting points of each product (Wong & Medrano, 2005). A single peak shows high specificity of the test, as only one PCR product is being made.



### **3. Results**

#### ***Survey Data***

27 farmers agreed to receive surveys, of which 10 replied. Figure 1 shows the proportion of farmers keeping different types of cattle, with spring-born suckler calves being kept by 60%, followed by suckler cows and dairy beef growing cattle, each being kept on 50% of farms. Farmers who graze their cattle typically turn out in April, rehousing around November. 60% of farmers employ rotational grazing, of which 83% rotated between different ages of cattle and 50% rotated between different grazing species. 70% graze their 1<sup>st</sup> year cattle on the same area of pasture each year and 70% reported spreading muck/slurry on pasture grazed by cattle. 80% had a worm control policy in place. 50% use anthelmintics only, 12.5% use pasture management only and 25% use a combination of anthelmintics and pasture rotation.

Of the 80% of farmers who routinely treat their cattle, 67% treated twice per year, the remaining farmers treating once. Pour-on macrocyclic lactones were the most widely used products, used on 75% of farms (Figure 2). The average interval between switching to a new group of anthelmintics was 2-3 years (57%), although 29% reported never changing anthelmintic group (Figure 3). When asked how they arrived at their current worm control policy, 43% stated veterinary advice, 14% relied on information from the drug manufacturers, 14% used advice from other farmers. The remaining 29% claimed to develop their programme in other ways. 25% of farmers treat their cattle before turnout, 50% treat before housing and 25% treat before both turnout and housing. 56% treat in the first grazing season only (Figure 4) and 38% of those who treat use a dose and move strategy.

40% of farmers buy in replacement cattle, but only 60% of them worm and quarantine the new animals before mixing them into the existing herd. None of the farmers surveyed had previously had any tests for the presence of AR nematodes on their farm and none routinely treated dry cows.

#### ***Faecal Egg Counts***

Samples were received from 16 farms and egg counts were performed on the day of arrival. As shown by Figure 5, the number of eggs found varied between the farms, ranging from 20 epg to 280epg, of which half exceeded 100epg.

### **qPCR**

The qPCR was highly effective at detecting and amplifying DNA from L1 larvae. Detection of *Ostertagia* was insufficient when 1µl of DNA template was used with the Ct values falling around 35, close to the total number of cycles (40), although *Cooperia* appears to be detected well at this concentration (average Ct= 18.3) (Table 1, Figure 8). When 5µl of DNA template was used both species were detected well, with Ct values averaging 16 for *Cooperia* and 14.5 for *Ostertagia* (Table 2, Figure 9). The average ratio of *Cooperia* to *Ostertagia* was approximately 1:1.5, although there was high variation between individual farms (Figure 6). The dissociation curves for the *Cooperia* (Figure 10) and *Ostertagia* (Figure 11) each show a single peak, indicating high specificity.

## **4. Discussion**

### **Surveys**

The survey shows farmers are still using anthelmintic programmes and management practices known to accelerate the development of AR in GI nematodes, such as dosing and moving animals. Half the farmers asked are relying on anthelmintics to control GI nematodes, with pour-on ML products being the most common worming product in use, despite evidence it promotes AR due to its variable efficacy and slowly declining drug concentrations (Coles, 1988). Only 14% changed the products used on an annual basis as recommended, leaving a large number of farmers who rarely or never change anthelmintic groups (Barnes *et al.*, 1995). This prolonged exposure of nematodes to the same types of anthelmintics greatly favours resistance to these drugs as resistant individuals are given a significant advantage over susceptible individuals. Less than half the farmers asked used veterinary advice to develop their worming programme, which could be linked to the general lack of veterinary input, especially on beef farms, as low profit margins discourage farmers from seeking costly veterinary input.

Previous research of farming practices in the South West area found 65% of farmers grazing FGS on the same area of pasture each year, similar to the 70% who reported doing so in the current study (Stafford & Coles, 1999). The number of farmers spreading muck on their grazing land has fallen slightly, from 77% to 70% but generally results show fairly little change from surveys conducted 15 years ago (Stafford & Coles, 1999). However, farmers reported far less frequent use of anthelmintics, all treating

twice a year or less compared to 52% treating 3 or more times per year in 1999, and changing anthelmintics groups more often, with only 29% having never changed anthelmintic groups compared to the 53% found previously (Stafford & Coles, 1999). Given the effort organisations such as EBLEX have put into informing farmers and creating accessible publications, greater improvements would have been hoped for.

### **FECs**

Egg counts of 100-200epg indicate a worm burden that may require attention, for example if the animals are scouring or if it is early in the grazing season, as pasture contamination would have time to rise to high levels by the end of the grazing season. Of the 16 farms sampled, 7 exceeded FECs of 100epg (Figure 5); as the cattle will typically have another 4-5 months at pasture, nematodes could be a factor to consider if any performance or health issues arise in these herds. Of the farmers who graze their FGS calves on the same area of pasture each year, the average FEC was 102epg, higher than those who reported using different pasture each year which averaged only 72epg, although the difference was not significant ( $p>0.05$ ).

### **qPCR**

The qPCR appears to be sufficiently sensitive to detect and quantify the amount of DNA template present in L1 larvae of *Cooperia* and *Ostertagia* when 5 $\mu$ l of DNA template is used, as indicated by the Ct values. No cross reactions between the species appear to occur, making the test highly species specific (Figures 9 and 10). By using L1 larvae, rather than L3 as in other studies, tests could be completed within 48 hours if necessary, significantly reducing the time and resources required to culture larvae.

On average, there were more *Ostertagia* (61%) found via qPCR in the samples than *Cooperia* (39%), although there was much variation between farms (SD= 26.5). Timing of anthelmintic treatment had no significant effect ( $p>0.05$ ) on the species present; the farms which only treated in the FGS had a higher proportion of *Cooperia* on average (55.2%), compared to farms that treated in both the FGS and the SGS, which had a higher proportion of *Ostertagia* (62%) on average. When FECs and the proportions of nematodes for each sample were plotted against each other  $r^2= 0.0032$ , indicating very little correlation between the number of eggs being shed and the species present (Figure 7).

In order for the ratios of species identified by the qPCR to reflect the true proportions of species in the host there must be equally efficient recovery rates of eggs and hatching of larvae from each species (Taylor *et al.*, 2002). In addition the results of qPCR assume the size and therefore the amount of DNA present in each individual at a given developmental stage, both within and between species, are the same (Höglund *et al.*, 2013). Previous studies have shown a high level of agreement between qPCR and methods such as microscopy (Gasser, 1999).

Whilst these techniques can give highly useful information and play an important role in the diagnosis and monitoring of nematode disease, the individual circumstances of the farms and animals being tested must be taken into account as this strongly influences the way data should be interpreted and used. Factors such as the condition of the animal or time of year can determine whether a result indicates a serious problem that requires immediate action or simply normal variation that is expected and does not pose a threat to animal health and performance.

## **5. Conclusion**

The results of the survey raise questions as to how well the information gained through research is being communicated to farmers. Ultimately it is farmers that have the biggest influence over how well sustainable worming programmes are accepted and implemented as they are the end users of products and programmes developed through research. As such, it should be the utmost priority of veterinarians those carrying out research to ensure farmers are receiving correct, up to date information to allow them to make suitable decisions about how they manage their animals. Educating farmers will encourage them to make parasite control decisions with long term sustainability in mind, rather than what is the cheapest or simplest to perform. The use of molecular techniques in conjunction with microscopy can provide valuable information for vets and researchers who wish to closely monitor changes in the nematode population either within individual hosts or at a herd level. The relatively short timescale required to use FECs and qPCR together makes them suitable for clinical diagnostic testing, and such techniques could form part of herd health monitoring to identify signs of developing AR before it becomes an unmanageable problem. These tests are best used to help inform a diagnosis, taking into account the

individual situation, or as part of a continual monitoring programme, than as standalone measurements of disease.

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## Tables and Figures

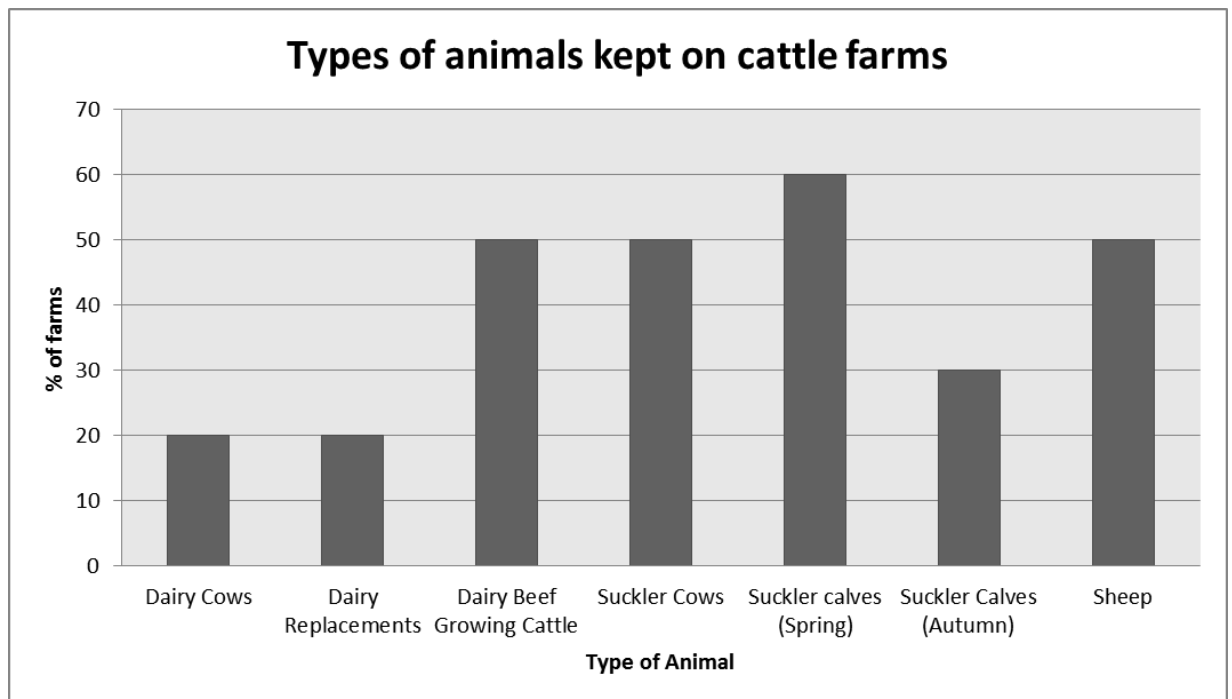


Figure 1. Types of animals kept by the farmers surveyed.

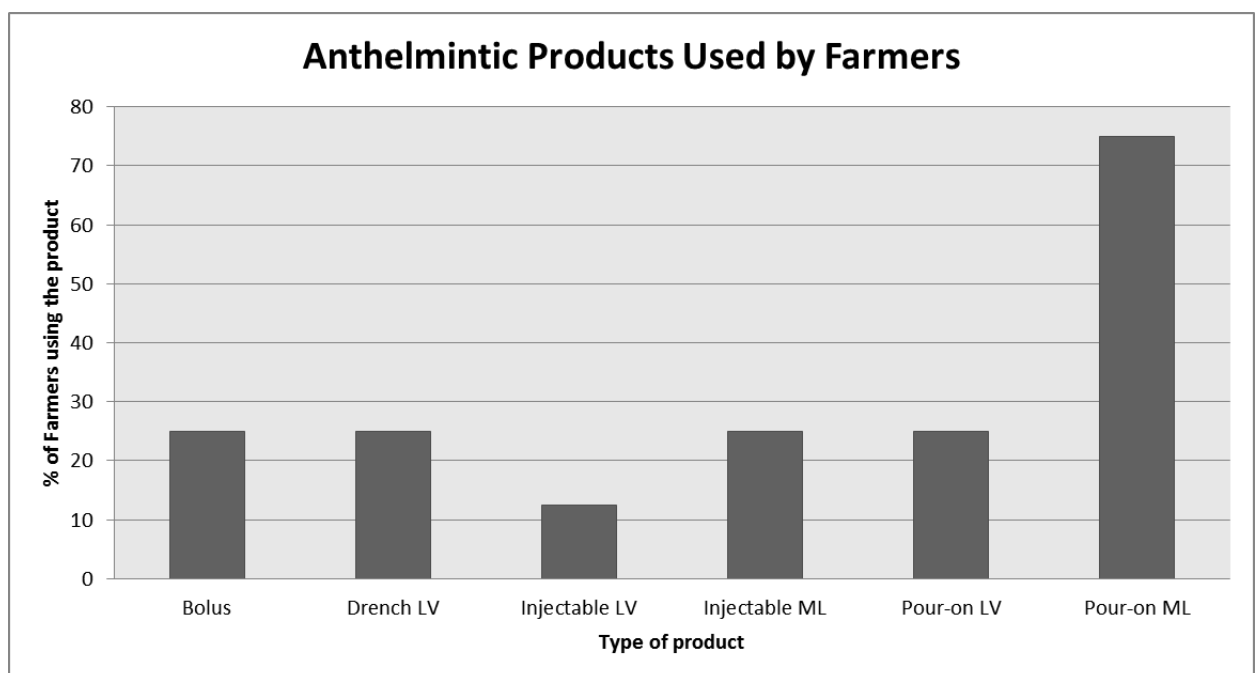


Figure 2. The types of anthelmintic products used by farmers to worm their cattle. LV- Levamisole. ML- Macrocytic lactones. Boluses are benzimidazole based.



### Typical interval between changing anthelmintic groups

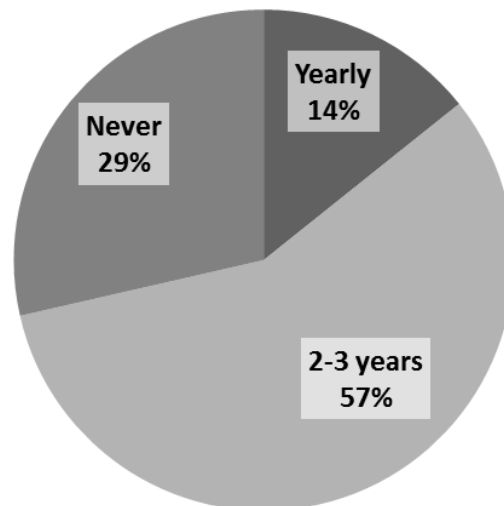


Figure 3. Interval between changing anthelmintics from one drug group to another.

### Timing of anthelmintic treatment

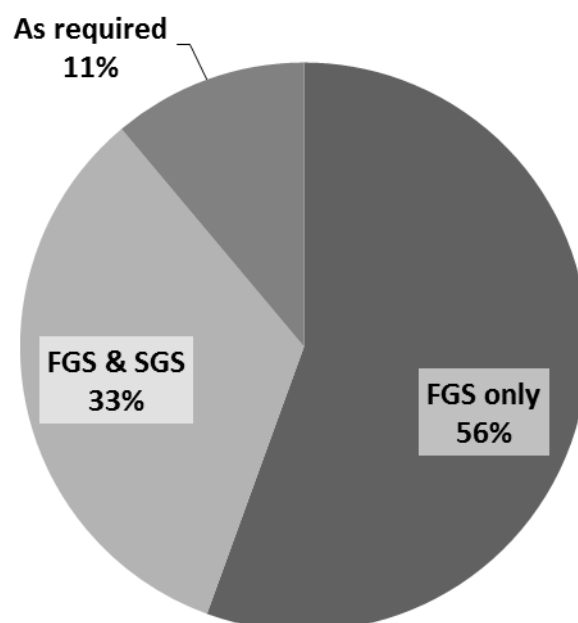


Figure 4. Typical timing of anthelmintic treatments. FGS- First Grazing season. SGS- Second Grazing season.

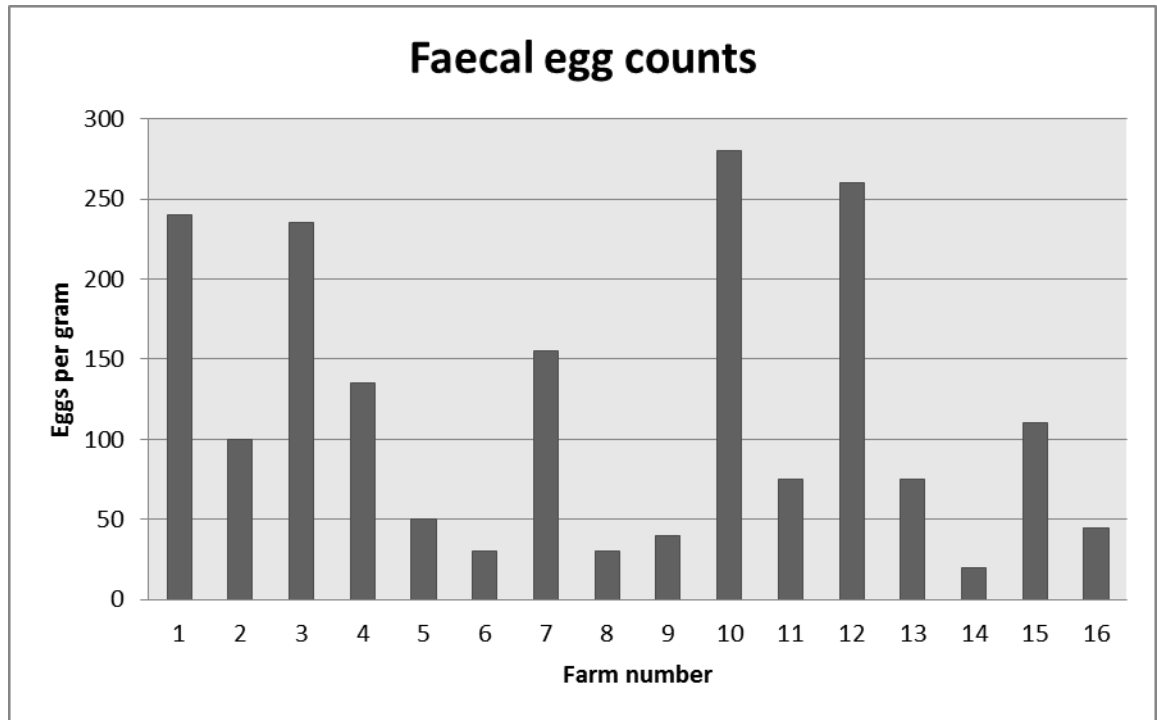


Figure 5- Eggs per gram of faeces from each farm sampled.

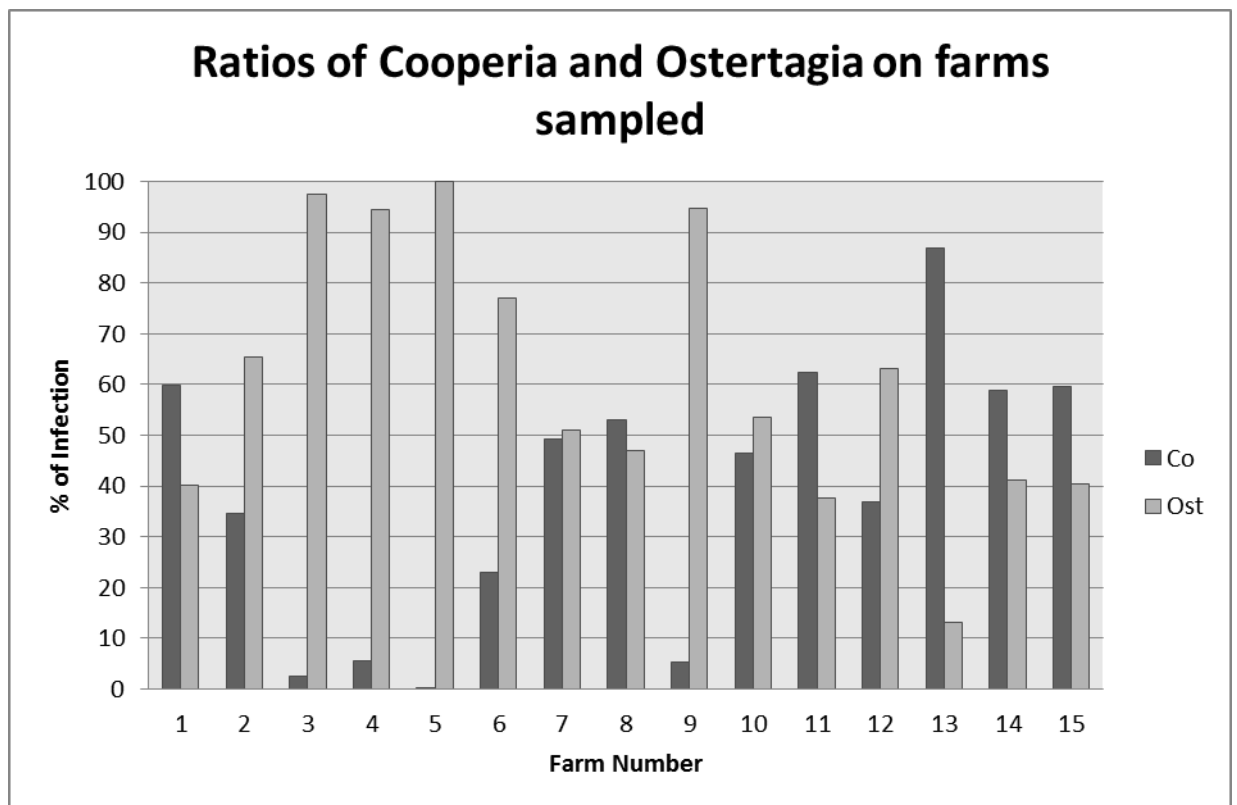


Figure 6. Proportions of *Cooperia* (Co) and *Ostertagia* (Ost) hatched to L1 from eggs in faecal samples from farms.

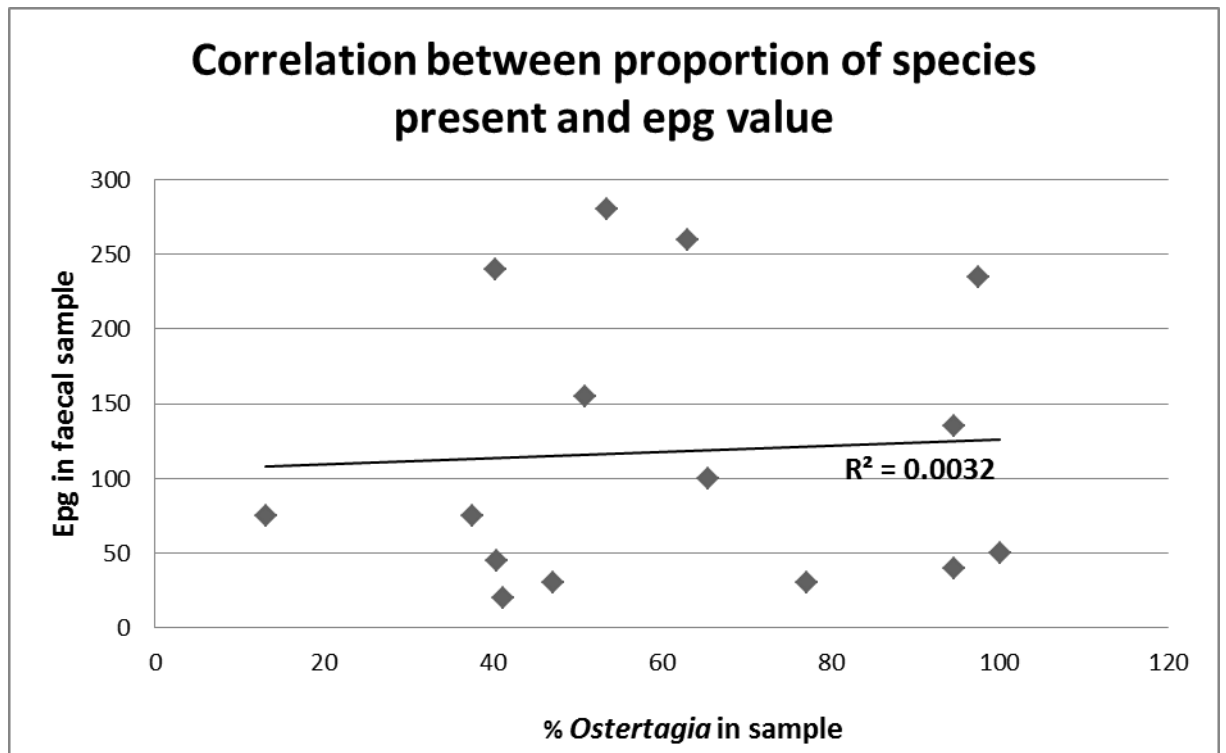


Figure 7. Correlation between the species present in a sample and epg.  $r^2=0.0032$ .



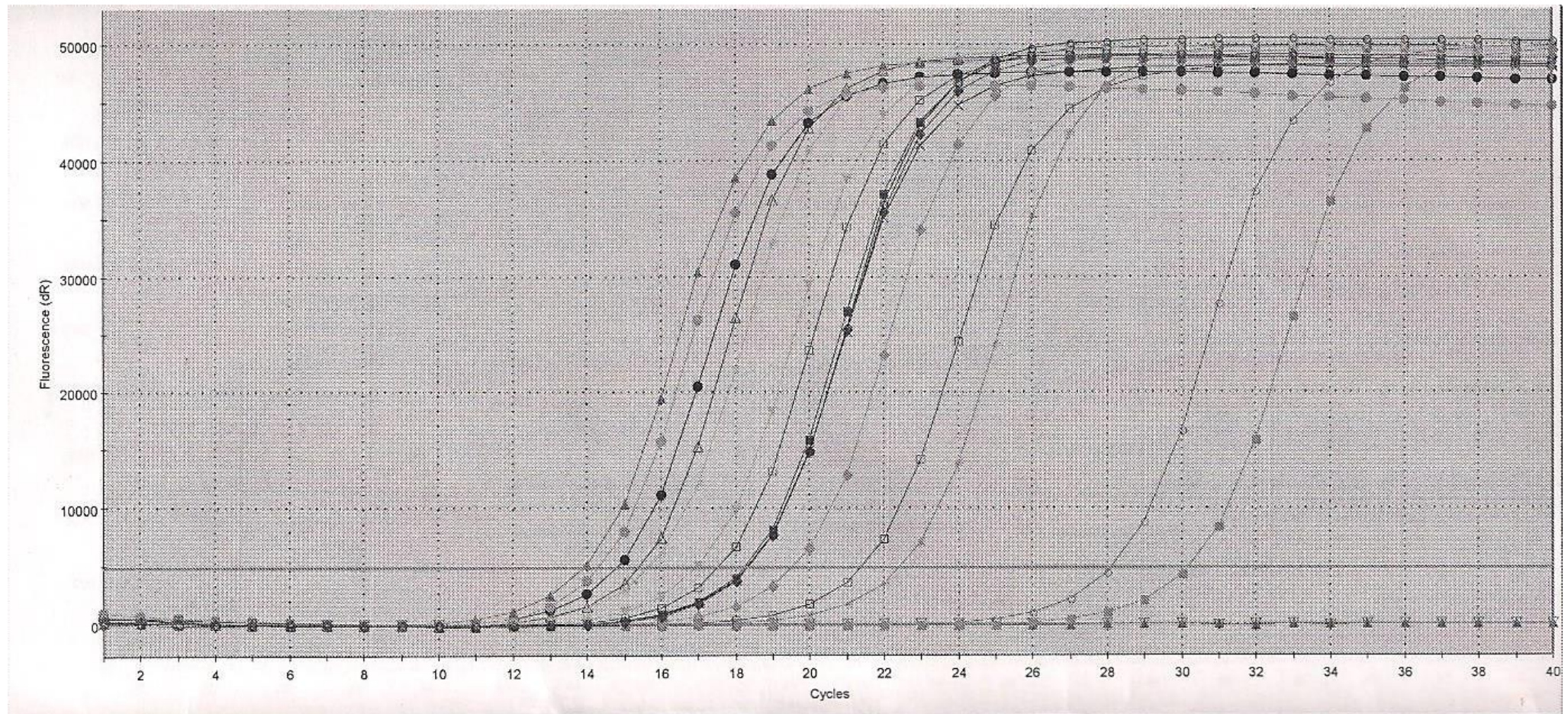


Figure 8. Amplification curve for *Cooperia* at 1 $\mu$ l DNA



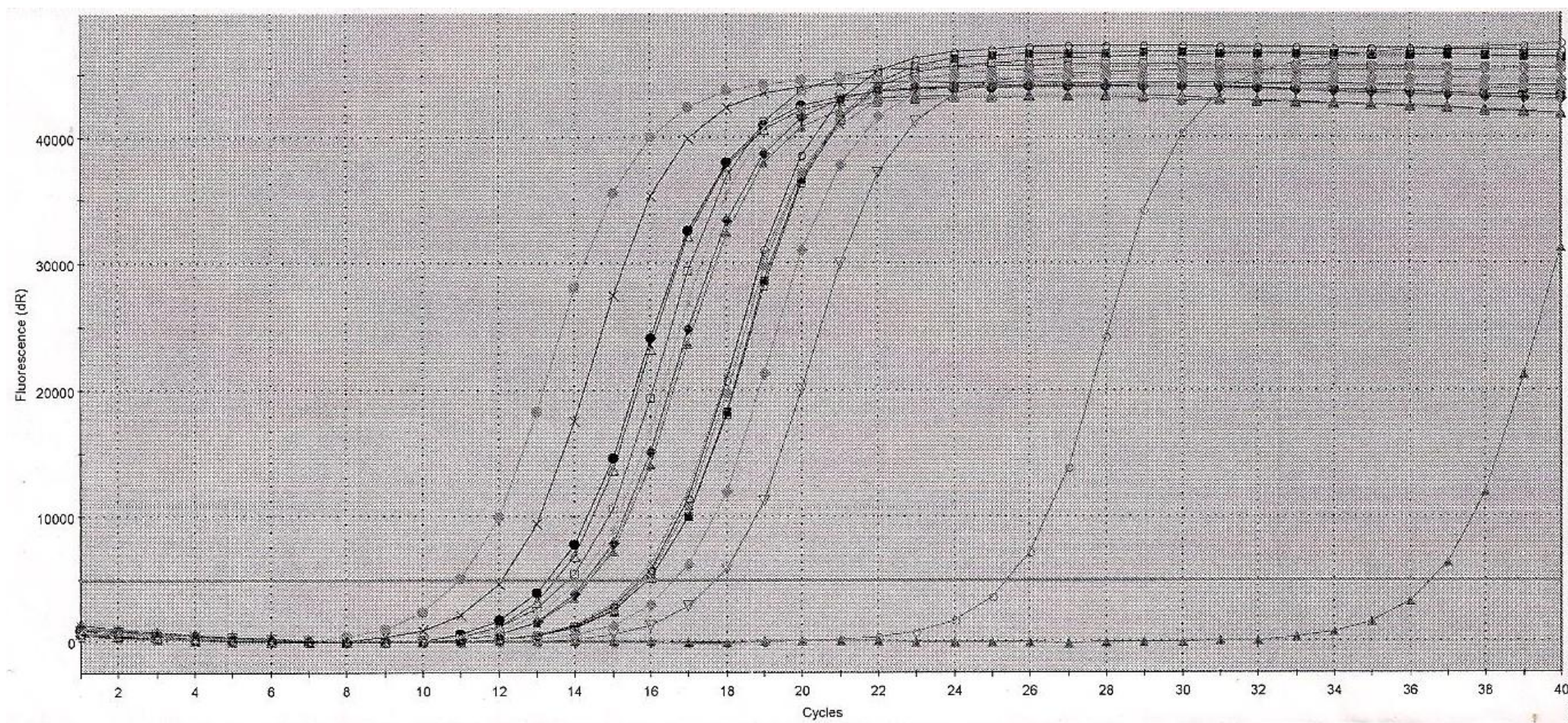


Figure 9. Amplification curve for *Ostertagia* at 5 $\mu$ l DNA.



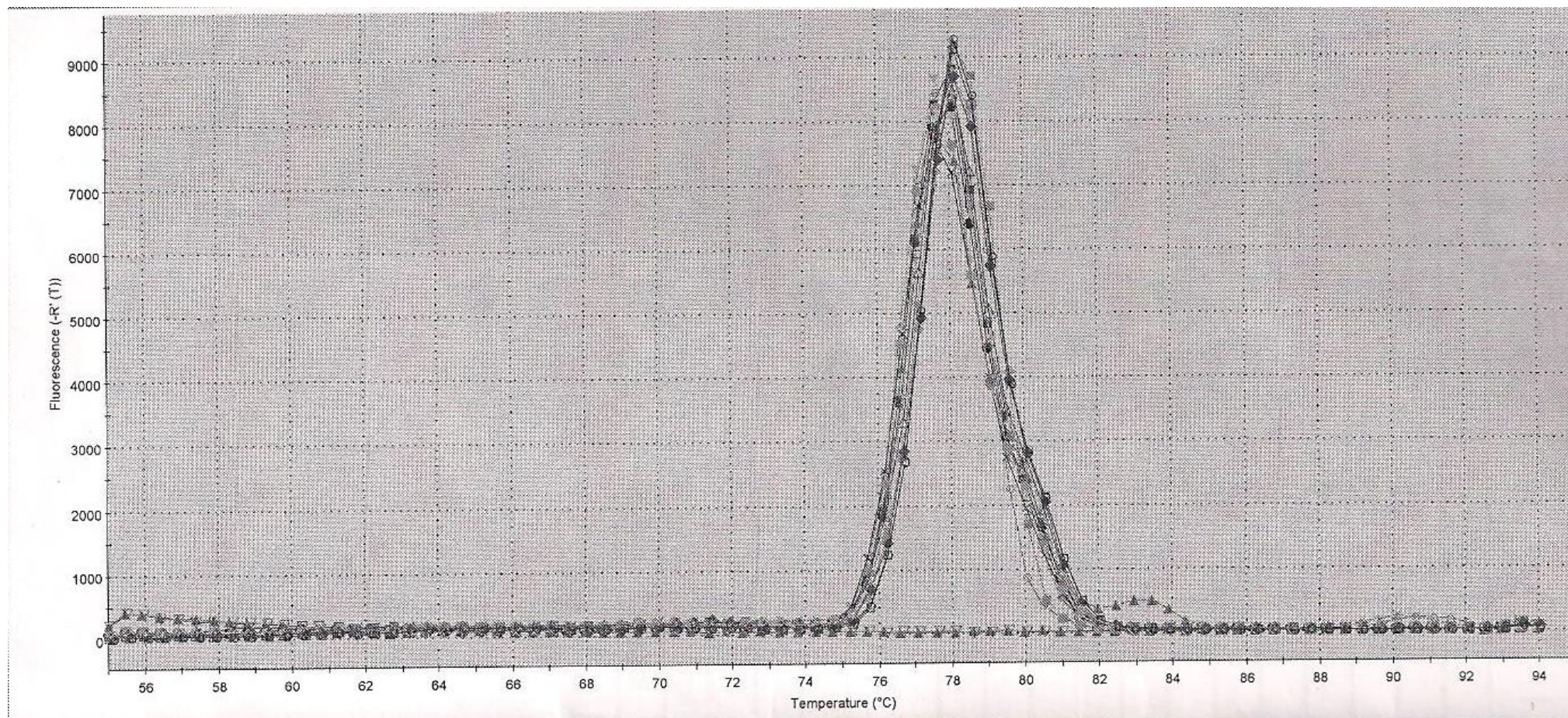


Figure 10. Dissociation curve for *Cooperia*.



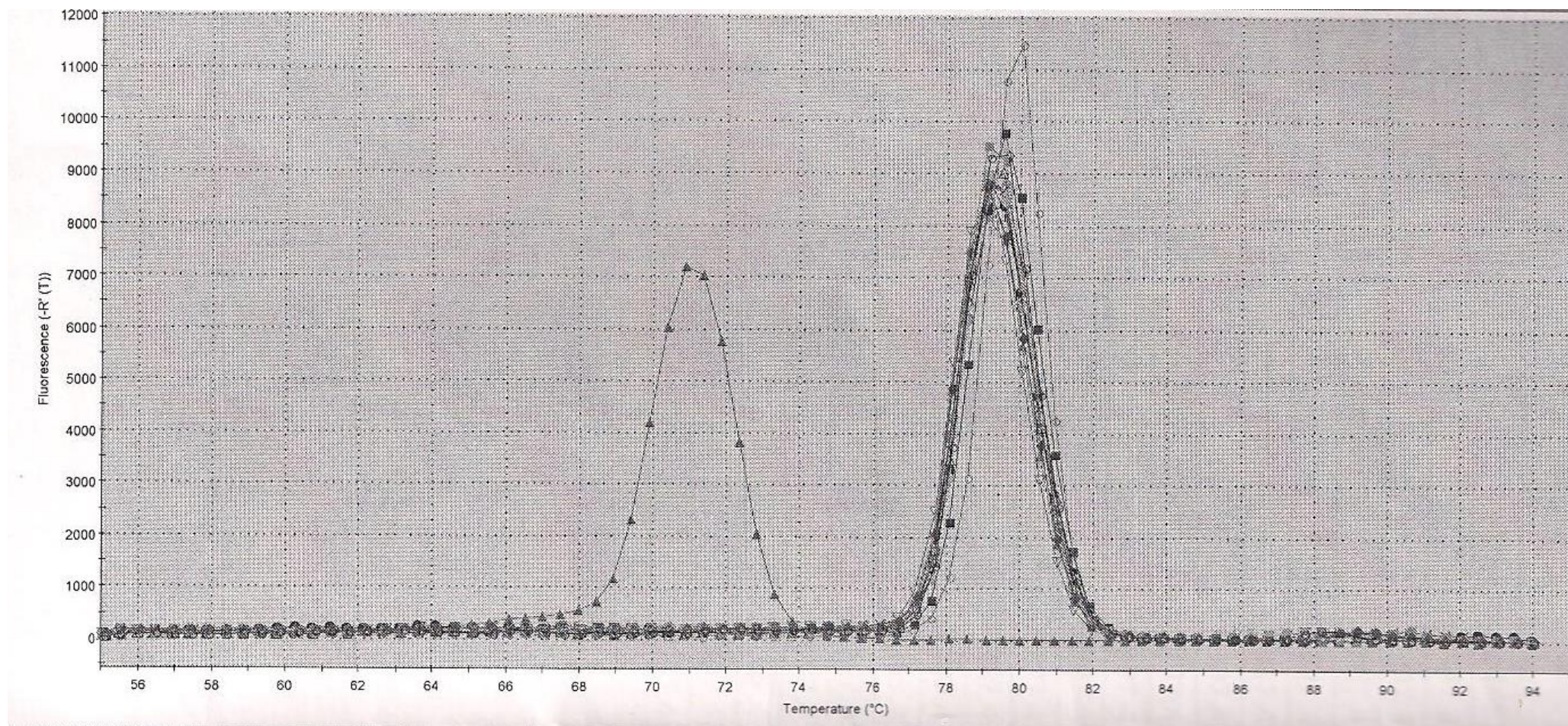


Figure 11. Dissociation curve for *Ostertagia*. Peak at 71°C due to negative control.



Table 1- Ct values and proportions of *Ostertagia* and *Cooperia* detected when using 1µl DNA.

Farm Number	Ct Cooperia	Ct Ostertagia	% Cooperia	% Ostertagia
1	14.83	35.48	1.00E+02	6.08E-05
2	19.59	35.94	1.00E+02	1.20E-03
3	21.43	34.88	1.00E+02	8.94E-03
4	18.33	35.74	1.00E+02	5.74E-04
5	30.19	33.94	9.31E+01	6.92E+00
6	18.38	35.43	1.00E+02	7.37E-04
7	18.34	34.27	1.00E+02	1.60E-03
8	18.26	34.95	1.00E+02	9.46E-04
9	22.45	38.91	1.00E+02	1.11E-03
10	16.97	35.86	1.00E+02	2.06E-04
11	15.43	34.59	1.00E+02	1.71E-04
12	14.35	33.87	1.00E+02	1.33E-04
13	13.92	38.51	1.00E+02	3.96E-06
14	15.68	37.64	1.00E+02	2.45E-05
15	17.59	36.97	1.00E+02	1.47E-04

Table 2- Ct values and proportions of *Ostertagia* and *Cooperia* detected when using 5µl DNA.

Farm Number	Ct Cooperia	Ct Ostertagia	% Cooperia	% Ostertagia
1	12.77	13.34	59.75	40.25
2	17.61	16.69	34.58	65.42
3	19.12	13.85	2.53	97.47
4	16.19	12.08	5.47	94.53
5	28.17	15.83	0.02	99.98
6	16.10	14.35	22.92	77.08
7	15.82	15.77	49.13	50.87
8	15.78	15.95	52.94	47.06
9	19.81	15.66	5.33	94.67
10	14.62	14.42	46.54	53.46
11	12.83	13.56	62.39	37.61
12	11.75	10.98	36.96	63.04
13	11.73	14.46	86.90	13.10
14	13.63	14.14	58.75	41.25
15	15.39	15.95	59.58	40.42