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**DOTTORATO DI RICERCA IN  
ORGANISMI MODELLO NELLA RICERCA BIOMEDICA E VETERINARIA**

**- XXVII CICLO -**

**MINI-FLOTAC:**

**A NEW TOOL FOR DIAGNOSIS OF NEMATODES IN SHEEP**

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*"Il mondo è nelle mani di coloro che hanno il coraggio di sognare e di correre il rischio di vivere i propri sogni."*

*- Paulo Coelho -*

*"The world is in the hands of those who have the courage to dream and run the risk of living their dreams"*

*- Paulo Coelho -*

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## LIST OF ABBREVIATIONS

AR	Anthelmintic resistance
CI	Confidence interval
CPG	Cysts per gram of faeces
CREMOPAR	Centro Regionale Monitoraggio Parassitosi (Regional Center Monitoring of Parasitic Infections), Campania region, southern Italy
CV	Coefficient of Variation
DALP	Department of Agriculture and Livestock Production of the Campania region (southern Italy)
DISCONTTOOLS	Disease Control Tools
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram of faeces
EU	European Union
FCS	Faecal consistency score
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
FP	Framework Programme
FS	Flotation solution
FS1	Sheather's sugar solution
FS2	Saturated sodium chloride
FS3	Zinc sulphate
FS4	Sodium nitrate
FS5	Sucrose and potassium iodomercurate
FS6	Magnesium sulphate
FS7	Zinc sulphate
FS8	Potassium iodomercurate
FS9	Zinc sulphate and potassium iodomercurate
GIN	Gastrointestinal nematodes
GI strongyles	Gastrointestinal strongyles



GLM	Generalized linear model
GLOWORM	Innovative and sustainable strategies to mitigate the impact of global change on helminth infections in ruminants (FP7) Project KBBE-2011-5- 288975
IVM	Ivermectin
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
LC	Larval culture
LCL	Lower confidence limit
LPG	Larvae per gram of faeces
LSD	Least significant difference
LV	Imidazothiazoles/Tetrahydropyrimidines
McM	McMaster
MT-PCR	Multiplexed Tandem PCR
OPG	Oocysts per gram of faeces
PCR	Polymerase Chain Reaction
PGE	Parasitic gastroenteritis
PP	Periparturient period
PPR	Peri-parturient rise
qPCR	Quantitative Polymerase Chain Reaction
SD	Standard deviation
RT-PCR	Real-time Polymerase Chain Reaction
SCOPS	Sustainable Control of Parasites in Sheep
SG	Specific gravity
SOP	Standard operating procedures
TST	Targeted selective treatment
TT	Targeted treatment
UNINA	University of Naples Federico II
WAAVP	World Association for the Advancement of Veterinary Parasitology

## **GENERAL INTRODUCTION**

## **I. THE IMPORTANCE OF GASTROINTESTINAL NEMATODES IN SMALL RUMINANTS**

Small ruminant farming has a prominent role in the sustainability of rural communities around the world (Park and Haenlein, 2006), as well as being socially, economically and politically highly significant at national and international levels, as with all livestock species (Morgan et al., 2013). In the European Union (EU), for instance, there are currently around 101 million sheep and 12 million goats (FAOSTAT, 2009). Efficient small ruminant livestock production is also crucial to meet the increasing demands of meat and dairy products, especially in areas in which land is unsuitable for growing crops (Chiotti and Johnston, 1995). Small ruminant dairying is particularly important to the agricultural economy of the Mediterranean region, which produces 66% of the world's sheep milk and 18% of the world's goat milk (Pandya and Ghodke, 2007).

However, there are several factors which affect the productivity of the small ruminant livestock sector, the capacity to maintain and improve a farm (i.e. its health and genetic potential) and, as a consequence, also human nutrition, community development and cultural issues related to the use of these livestock species (Perry and Randolph, 1999; Nonhebel and Kastner, 2011).

Among the factors that negatively affect the livestock production, infections with parasites and in particular with gastrointestinal nematodes (GIN) continue to represent a serious challenge to the health, welfare, productivity and reproduction of grazing ruminants throughout the world (Morgan et al., 2013).

All grazing animals are exposed to helminth infections at pasture and any respective future intensification of livestock farming will increase the risk of helminth infections/diseases (Morgan et al., 2013). The ranking of GIN as one of the top cause of lost productivity in small and large ruminants by the recent DISCONTTOOLS programme (<http://www.discontools.eu/home/index>) reinforces the increasing EU's consideration of the impact of these parasites upon animal health, welfare and productivity (Vercruysse, personal communication).

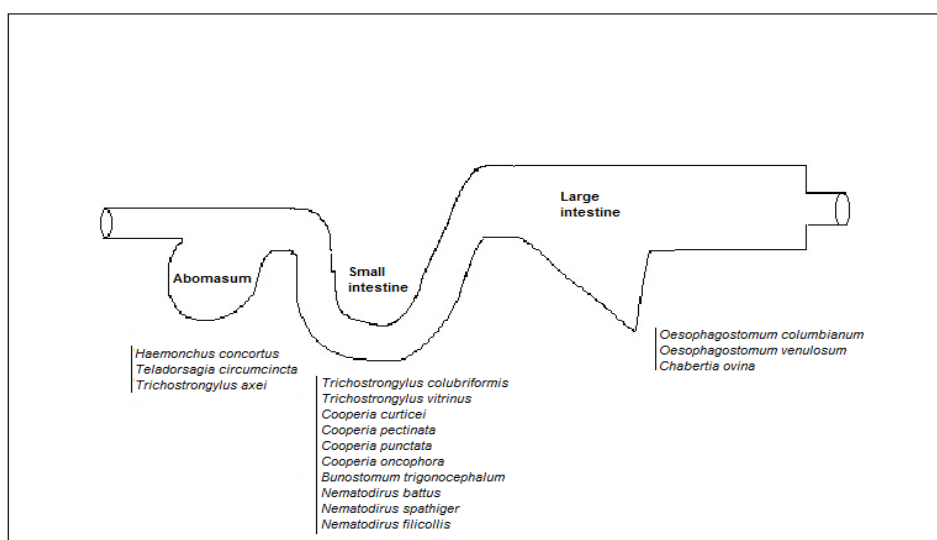
The economic costs of parasitic infections are currently difficult to quantify, however some estimates do exist within the scientific literature; for example,

studies in the UK have estimated the cost of GIN infections of sheep to be in the order of 99m € per year (Nieuwhof and Bishop, 2005).

Within the EU as a whole, annual sales of anthelmintic drugs used to control these infections in ruminants have been estimated to be in the order of 400 million € (Selzer, 2009). It is likely that these figures only represent the tip of the iceberg when it comes to calculating the true cost of livestock helminthoses endemic within the EU (Charlier et al., 2009).

## II. LIFE CYCLE AND EPIDEMIOLOGY OF GASTROINTESTINAL NEMATODES IN SMALL RUMINANTS

Grazing ruminants are frequently parasitized by multiple species of GIN (Nematoda, Strongylida, Trichostrongylidea), also known as gastrointestinal (GI) strongyles, which cause the so-called parasitic gastroenteritis (PGE) (Kassai, 1999). With respect to small ruminants, GIN parasitizing the abomasum, small and large intestines of sheep and goats include species of *Haemonchus*, *Ostertagia* (*Teladorsagia*), *Trichostrongylus*, *Nematodirus*, *Oesophagostomum*, *Chabertia* and *Bunostomum* (Zajac, 2006) listed in the following Figure 1.



**Fig. 1.** Location in the host of the prevalent species of GIN infecting small ruminants in Europe.

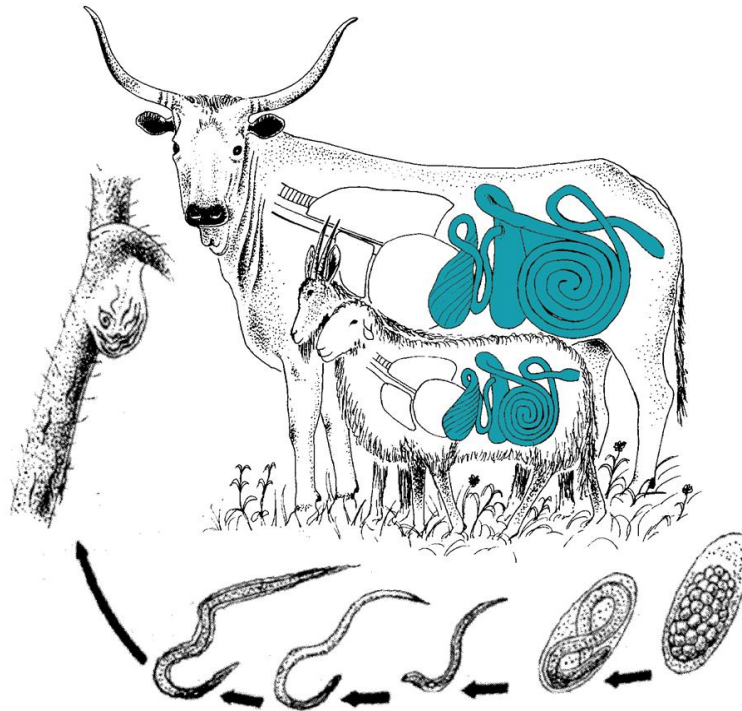
Some key morphological characteristics (length), pre-patent period (days) and location in the host of the genera of GIN that infect small ruminants in Europe are listed in the following Table 1.

**Table 1.** The length, pre-patent period and location in the host of the most important genera of GIN infecting sheep in Europe (from Anderson, 2000; Taylor et al., 2007; Roeber et al., 2013a).

Genus	Length (mm)	Pre-patent period (days)	Location in the host
<i>Haemonchus</i>	♂ 10-20 ♀ 18-30	18-21	Abomasum
<i>Teladorsagia</i>	♂ 7-8 ♀ 10-12	15-21	Abomasum
<i>Trichostrongylus</i>	♂ 2-8 ♀ 3-9	15-23	Abomasum or small intestine
<i>Cooperia</i>	♂ 4-5 ♀ 5-6	14-15	Small intestine
<i>Nematodirus</i>	♂ 10-19 ♀ 15-29	18-20	Small intestine
<i>Bunostomum</i>	♂ 12-17 ♀ 19-26	40-70	Small intestine
<i>Oesophagostomum</i>	♂ 12-16 ♀ 14-24	40-45	Large intestine
<i>Chabertia</i>	♂ 13-14 ♀ 17-20	42-50	Large intestine

In general, with some exceptions (e.g. *Nematodirus*, *Bunostomum*), the life cycle of the GIN genera listed in Table 1 follows a similar pattern (Levine, 1968) as shown in Figure 2. Sexually dimorphic adults are present in the digestive tract, where fertilized females produce large numbers of eggs which are passed in the faeces. Strongyloid eggs (70–150 µm) usually hatch within 1–2 days. After hatching, larvae (L1) feed on bacteria and undergo two moults to then develop to ensheathed third-stage larvae (L3s) in the environment (i.e. faeces or grass). The sheath (which represents the cuticular layer shed in the transition from the L2 to L3 stage) protects the L3 stage from environmental conditions but prevents it from feeding. Infection of the host occurs by ingestion of L3s (with the exception of *Nematodirus* for which the infective L3 develops within the egg and of *Bunostomum* for which L3s may penetrate through the skin of the host). During its passage through the stomach, the L3 stage loses its protective sheath and has a histotrophic phase (tissue phase), depending on species, prior to its transition into the L4 and adult stages (Levine, 1968). Under unfavourable conditions, the larvae undergo a period of hypobiosis (arrested development; typical for species of *Haemonchus* and *Teladorsagia*); hypobiotic larvae usually resume their activity and development in spring in the case of *Haemonchus* or autumn in the case of *Teladorsagia* (Gibbs, 1986). This may be

synchronous with the start of the lambing season, manifesting itself in a peri-parturient increase in egg production in ewes (Salisbury and Arundel, 1970). The peri-parturient reduction of immunity increases the survival and egg production of existing parasites, increases susceptibility to further infections and contributes to the contamination of pasture with L3s when young, susceptible animals begin grazing (Hungerford, 1990).



**Fig.2.** The life-cycle of most genera and species of GIN in ruminants.

The importance of different genera/species of GIN as causes of disease in small ruminants depends not only on their presence, but also on their abundance (number of conspecific parasites living in a host) and seasonal patterns of infection. The large number of prevalence surveys and studies of field epidemiology in diverse regions provide a picture of the distribution and relative importance of different species of GIN in Europe. In line with the distribution in the southern hemisphere (Kao et al., 2000), *H. contortus* tends to be more common and more threatening to sheep health and production in warmer, southern areas, while *T. circumcincta* is the dominant nematode species of sheep in temperate and northern regions. *Trichostrongylus* and *Nematodirus* spp. are ubiquitous and their importance varies at local scale. *N. battus* is a major cause of disease in lambs only in northern Europe (Morgan and van Dijk, 2012). Follow-up prevalence data on GIN genera in sheep in Europe have been recently generated within the EU-FP7 GLOWORM project

(Innovative and sustainable strategies to mitigate the impact of global change on helminth infections in ruminants). The following Table 2 reports the prevalence data of GIN from 3 key European regions (Italy, Switzerland and Ireland).

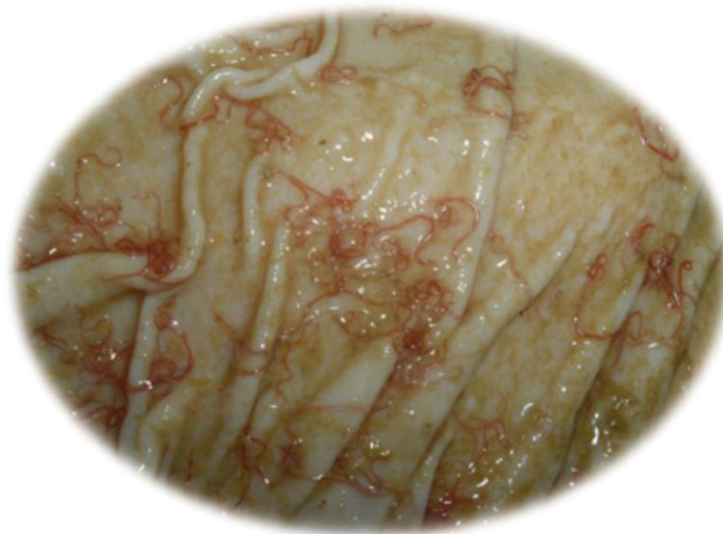
**Table 2.** The prevalence of the most important genera of GIN infecting sheep in Europe (Musella et al., 2011; Dipineto et al., 2013; EU-FP7 GLOWORM Project - www.gloworm.eu).

<b>GIN genera</b>	<b>Italy (no. farms tested = 139) Prevalence Min-Max (%)</b>	<b>Switzerland (no. farms tested = 133) Prevalence Min-Max (%)</b>	<b>Ireland (no. farms tested = 103) Prevalence Min-Max (%)</b>
<i>Haemonchus</i>	56.3 – 72.4	71.6 – 81.7	3.6 – 6.1
<i>Teladorsagia</i>	93.8 – 100	73.1 – 85.9	92.9 – 97.0
<i>Trichostrongylus</i>	93.8 – 96.6	89.5 – 93.9	89.3 – 97.0
<i>Cooperia</i>	12.5 – 34.5	28.2 – 32.8	33.3 – 60.7
<i>Nematodirus</i>	35.1 – 53.8	33.3 – 38.9	61.0 – 68.8
<i>Bunostomum</i>	0 – 3.4	0 – 8.5	3.6 – 9.1
<i>Oesophagostomum/ Chabertia</i>	81.3 – 89.7	56.7 – 83.1	3.6 – 97.0

### III. PATHOGENESIS AND PATHOLOGY OF GASTROINTESTINAL NEMATODES IN SMALL RUMINANTS

Different species of GIN can vary considerably in their pathogenicity, geographical distribution, prevalence and susceptibility to anthelmintics (Dobson et al., 1996). Mixed infections, involving multiple genera and species are common in sheep and goats, and usually have a greater impact on the host than mono-specific infections (Wimmer et al., 2004). Depending on the number, species and burden of parasitic nematodes, common symptoms of PGE include reduced weight gain or weight loss, anorexia, diarrhoea, reduced production and, in the case of blood-feeding genera (e.g. *Haemonchus*), anaemia and oedema, due to the loss of blood and/or plasma proteins (Kassai, 1999). Usually, low intensities of infection do not cause a serious hazard to the health of ruminants and may be tolerated (i.e. allowing the

development of some immunity in the host), but as the numbers of worms increase, subclinical disease can manifest itself and is, therefore, of great economic importance (Fox, 1997; Zajac, 2006). The severity of diseases caused by GIN in ruminants is influenced by several factors such as: i) the parasite species - *H. contortus*, *T. circumcincta* and intestinal species of *Trichostrongylus* are considered highly pathogenic in sheep (Besier and Love, 2003); ii) the number of worms present in the gastrointestinal tract; iii) the general health and immunological status of the host; iv) environmental factors, such as climate and pasture type; v) other factors as stress, stocking rate, management and/or diet (Kassai, 1999). Usually, three groups of animals are prone to heavy worm burdens: (i) young, non-immune animals; (ii) adult, immuno-compromised animals; and (iii) animals exposed to a high infection pressure from the environment (Zajac, 2006). Beyond any doubt, a GIN species of primary concern is *H. contortus* (Fig. 3), a highly pathogenic blood-feeder helminth that causes anaemia and reduced productivity and can lead to death in heavily infected animals (Burke et al., 2007).



**Fig. 3.** An abomasum of a sheep highly infected by *H. contortus*.

#### **IV. CONCLUDING REMARKS AND NEEDS FOR RESEARCH**

Although representing a significant economic and welfare burden to the global ruminant livestock industry, GIN infections in small ruminants are often neglected and implementation in research, diagnosis and surveillance of these parasites is



still poor, mainly in the matter of diagnostic methods and their use/interpretation. The accurate diagnosis (and interpretation) of GIN infection directly supports parasite control strategies and is relevant for investigations into parasite biology, ecology and epidemiology (Roeber et al., 2013b). This aspect is now particularly important given the problems associated with anthelmintic resistance (AR) in GIN populations of small ruminants worldwide (Roeber et al., 2013 a,b).

Various methods are employed for the ante mortem diagnosis of GIN infections in small ruminants. These include the observation of clinical signs indicative of disease (although non-pathognomonic), coprological diagnosis (faecal egg count – FEC), biochemical and/or serological, and molecular diagnostic approaches (reviewed in Roeber et al., 2013a). However, still now, faecal egg count (FEC) techniques remain the most common laboratory methods for the diagnosis of GIN in small ruminants. Also for FEC, as for many other diagnostic procedures used in parasitology, widespread standardization of laboratory techniques does not exist, and most diagnostic, research and teaching facilities apply their own modifications to published protocols (Kassai, 1999). Although FEC techniques are regarded to be standard diagnostic procedures, there is a lack of detailed studies of their diagnostic performance, including the diagnostic sensitivity, specificity and/or repeatability (Roeber et al., 2013a). Furthermore, many aspects including physical (pre-analytic), laboratory (technical) and biological (host-parasite-related) parameters – which affect FEC of GIN in small ruminants, as well as interpretation of FEC results, have poorly been investigated so far.

These are the reasons that motivated me in choosing “The coprological diagnosis of gastrointestinal nematode infections in small ruminants” as topic of this thesis to help optimize the use and interpretation of FEC in small ruminants.

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## **CHAPTER 1**

*“The coprological diagnosis of gastrointestinal nematode infections in small ruminants”*

## 1.1. INTRODUCTION

Even in the present era of genomics, metagenomics, proteomics and bioinformatics (Roeber et al., 2013), diagnosis of gastrointestinal nematodes (GIN) in ruminants still relies predominantly on coprological examination (Cringoli et al., 2010; Demeler et al., 2013). Indeed, coproscopy (from the Greek words *κόπρος* = faeces and *-σκοπία* = examen), i.e. the analysis of faecal samples for the presence of parasitic elements (e.g. eggs of GIN) is the most widely used diagnostic procedure in veterinary parasitology (Cringoli et al., 2004). This is the so-called coproscopy *sensu stricto*, instead, coproscopy *sensu lato* is the detection of antigens and/or DNA in faecal samples by immunological (e.g. ELISA) or molecular (e.g. (q)PCR) methods. After foundation of copromicroscopy by C.J. Davaine in 1857, several copromicroscopic techniques (and devices) have been developed, each with its own advantages and limitations.

Copromicroscopic diagnosis of GIN infections in small ruminants can be either qualitative (thus providing only the presence/absence of GIN eggs) or quantitative, providing also the number of eggs per gram of faeces (EPG), the so-called faecal egg counts (FECs). Egg counting of GIN eggs in small ruminants and other livestock species is a challenging topic for research in veterinary parasitology. Indeed, FECs have four important purposes.

The *first* is to determine whether animals are infected by GIN and to estimate the intensity (in terms of EPGs in the infected animals) of infection (McKenna, 1987; McKenna and Simpson, 1987). The *second* is to assess whether animals need to be treated to improve their health with the resulting increase of productive performance (Woolaston, 1992). The *third* is to predict pasture contamination by helminth eggs (Gordon, 1967). The *fourth* is to determine the efficacy of anthelmintics (Waller et al., 1989) by faecal egg count reduction (FECR) tests as well as monitoring control programmes and guide control decision (Brightling, 1988).

For the reasons listed above, small ruminant veterinary practitioners and parasitologists should re-evaluate their attitude of “it’s only a faecal sample” and should therefore consider that a suitable diagnosis of GIN and a correct

interpretation of FECs are of fundamental importance for a sustainable farming of small ruminants.

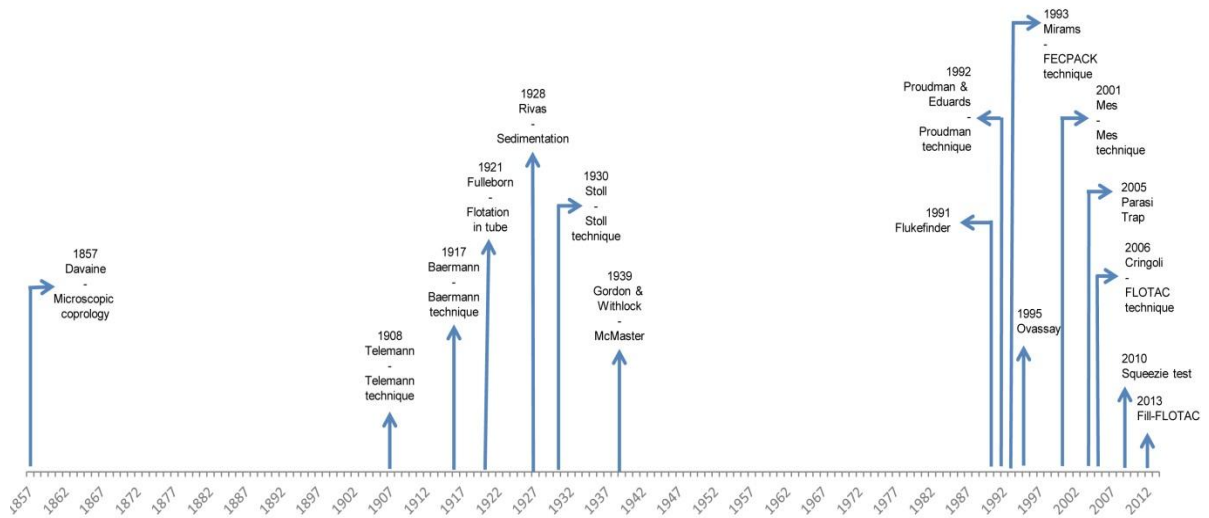
Chapter 1 provides an overview of the main egg counting methods used for GIN in small ruminants, with a particular focus on FEC techniques, the factors affecting their variability, as well as the use and interpretation of FEC results. The aim of this review is to consolidate information available in this important area of research and to identify some critical gaps in our current knowledge. Where information is lacking, suggestions are made as to how future research could improve our knowledge on the diagnosis of GIN infections in small ruminants.

The following sections of the chapter will provide detailed information and will evidence research gaps regarding:

- ✓ The operational and performance features of the main FEC techniques used in small ruminants for assessing GIN intensity and anthelmintic drug efficacy;
- ✓ The variability of the FEC techniques and the main factors – including physical (pre-analytic), laboratory (technical) and biological (host-parasite-related) parameters – which affect FECs of GIN in small ruminants; and
- ✓ The use and interpretation of FEC results, their significance and implications for both epidemiological surveys and control programmes.

## **1.2. COPROMICROSCOPIC TECHNIQUES : AN OVERVIEW**

Figure 1.1 reports a time chart showing the different copromicroscopic techniques (including devices) developed from 1857 to 2013, such as the direct centrifugal flotation method (Lane, 1922), the Stoll dilution technique (Stoll, 1923), the McMaster method (Gordon and Whitlock, 1939), the Wisconsin flotation method (Cox and Todd, 1962) and FLOTAC techniques (Cringoli et al., 2010, 2013).



**Fig. 1.1.** Time chart showing the different copromicroscopic techniques (including devices) developed from 1857 to 2013.

Most of the copromicroscopic techniques (some of which are still widely used) were developed between 1920 and 1940. After this twenty-year period, there has been a gap in research and no technique was developed until 1990. Afterwards, advances in developing copromicroscopic techniques occurred in the last 25 years (from 1990 to 2013) with the appearance of new diagnostic devices on the market. Remarkably, several manuals of diagnostic veterinary parasitology are available in the literature covering multiple animal species, including small ruminants, and describing a plethora of variants of the copromicroscopic techniques reported in Figure 1.1 (e.g. MAFF, 1986; Thienpont et al., 1986; Foreyt, 2001; Hendrix, 2006; Zajac and Conboy, 2012).

### 1.2.1. Sedimentation versus flotation

Qualitative and/or quantitative copromicroscopy in small ruminants usually involves concentration of parasitic elements (e.g. GIN eggs) by either sedimentation or flotation in order to separate GIN eggs from faecal material. The basic laboratory steps used to perform sedimentation and flotation methods are reported in the Appendix 1 and 2 of this chapter. It should be noted that several variants of these techniques are reported in literature.



The faecal sedimentation concentrates both faeces and eggs at the bottom of a liquid medium, usually tap water. In contrast, the principle of faecal flotation is based on the ability of a flotation solution (FS) to allow less dense material (including parasite eggs) to rise to the top. It should be noted that, in livestock species, sedimentation techniques are considered of less use (and time-consuming) to detect GIN eggs, whereas they are very useful for recovering heavy and operculated eggs (e.g. eggs of rumen and liver flukes, Paramphistomidae and *Fasciola hepatica*) that do not reliably float or are distorted by the effect of FS (Dryden et al., 2005). Thus, the methods most frequently used to recover GIN eggs in ruminant faeces are those based on flotation. These procedures are based on differences in the specific gravity of parasite eggs, faecal debris and FS.

### 1.2.2. Flotation solutions (FS)

Most of the FS used in coprology (see Table 1.1) are saturated and are made by adding a measured amount of salt or sugar (or a combination of them depending on the FS) to a specific amount of water to produce a solution with the desired specific gravity. After preparing any FS, it is mandatory to check the specific gravity with a hydrometer, recognizing that the specific gravity of the saturated solution will vary depending on ambient temperature. It should be noted that some of the FS listed in Table 1.1 contain ingredients that are harmful for humans and the environment (e.g. mercury II iodide) and hence they should be avoided if at all possible, especially in places with no or inappropriate waste control.

The FS used for copromicroscopic diagnosis of GIN infections in small ruminants are usually based on sodium chloride (NaCl) or sucrose and are characterized by a low specific gravity (usually 1.200).

It should be noted that the choice of FS is important but does not receive sufficient consideration by the scientific community, despite the substantial effect that the FS can have on the diagnostic performance of any flotation technique (Cringoli et al., 2004). Usually, in the manuals of diagnostic parasitology or in the peer-reviewed literature, only the specific gravity is reported for FS. It is commonly believed that the efficiency of a FS in terms of the capacity to bring eggs to float increases as the specific gravity of the FS increases. However, parasitic eggs should not be considered “inert elements” (Cringoli et al., 2004). Instead, interactions between

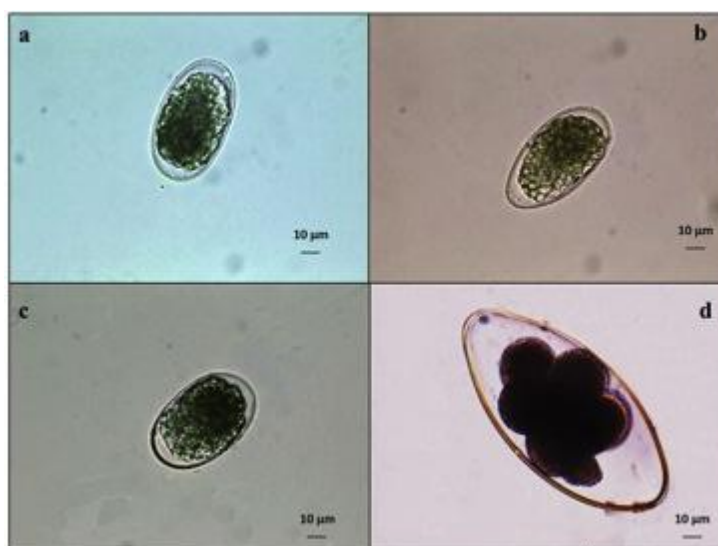
the elements within a floating fecal suspension (e.g., FS components, eggs and residues of the host alimentation) might be complex and new research is needed to elucidate potential interactions between these elements. Therefore, calibration of FEC techniques, to determine the optimal FS and faecal preservation method for an accurate diagnosis of parasitic elements, is a challenging topic of research.

**Table 1.1.** Flotation solutions (composition and specific gravity) most commonly used for copromicroscopy in small ruminants. Sodium chloride (in gray) is widely employed for flotation of GIN in ruminants.

<b>Flotation solution</b>	<b>Composition</b>	<b>Specific gravity</b>
Sucrose and formaldehyde	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> 454 g, CH <sub>2</sub> O solution (40%) 6 ml, H <sub>2</sub> O 355 ml	1.200
Sodium chloride	NaCl 500 g, H <sub>2</sub> O 1000 ml	1.200
Zinc sulphate	ZnSO <sub>4</sub> ·7H <sub>2</sub> O 330 g, H <sub>2</sub> O brought to 1000 ml	1.200
Sodium nitrate	NaNO <sub>3</sub> 315 g, H <sub>2</sub> O brought to 1000 ml	1.200
Magnesium sulphate	MgSO <sub>4</sub> 350 g, H <sub>2</sub> O brought to 1000 ml	1.280
Sodium nitrate	NaNO <sub>3</sub> 250 g, Na <sub>2</sub> O <sub>3</sub> S <sub>2</sub> ·5 H <sub>2</sub> O 300 g, H <sub>2</sub> O brought to 1000 ml	1.300
Zinc sulphate	ZnSO <sub>4</sub> ·7H <sub>2</sub> O 685 g, H <sub>2</sub> O 685 ml	1.350
Sodium chloride and zinc chloride	NaCl 210 g, ZnCl <sub>2</sub> 220 g, H <sub>2</sub> O brought to 1000 ml	1.350
Sucrose and sodium nitrate	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> 540 g, NaNO <sub>3</sub> 360 g, H <sub>2</sub> O brought to 1000 ml	1.350
Sodium nitrate and sodium thiosulphate	NaNO <sub>3</sub> 300 g, Na <sub>2</sub> O <sub>3</sub> S <sub>2</sub> ·5 H <sub>2</sub> O 620 g, H <sub>2</sub> O 530 ml	1.450
Sucrose and sodium nitrate and sodium thiosulphate	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> 1200 g, NaNO <sub>3</sub> 1280 g, Na <sub>2</sub> O <sub>3</sub> S <sub>2</sub> ·5 H <sub>2</sub> O 1800 g, H <sub>2</sub> O 720 ml	1.450

### 1.2.3. Identification of GIN eggs

From a general point of view, the main limitation of copromicroscopy for the diagnosis of GIN infections in small ruminants is based on the fact that for most GIN genera/species there is an overlap in size of the eggs (Fig. 1.2 a,b,c); only *Nematodirus* (Fig. 1.2 d) is an exception because its eggs are sufficiently different for their differentiation by size and shape (Table 1.2).



**Fig. 1.2.** GIN eggs (a,b,c) and *Nematodirus* egg (d).

**Table 1.2.** Morphometric characteristics of the eggs of different genera of GIN infecting small ruminants: size ( $\mu\text{m}$ ), shape and shell (data from Thienpont et al., 1986).

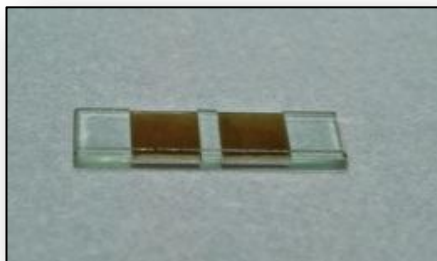
Genus	Size ( $\mu\text{m}$ )	Shape	Shell
<i>Haemonchus</i>	62-95 x 36-50	Oval; the eggs contain numerous blastomeres hard to distinguish	Thin
<i>Teladorsagia</i>	74-105 x 38-60	Oval; the eggs contain numerous blastomeres hard to distinguish	Thin
<i>Trichostrongylus</i>	70-125 x 30-55	Oval; the eggs contain 16 to 32 blastomeres	Thin
<i>Cooperia</i>	60-95 x 29-44	Oval with parallel sides; the eggs contain numerous blastomeres hard to distinguish	Thin
<i>Nematodirus</i>	152-260 x 67-120	Oval; the eggs contain numerous blastomeres hard to distinguish	Thin
<i>Bunostomum</i>	75-104 x 45-57	Oval; the eggs contain 4 to 8 blastomeres	Thin
<i>Oesophagostomum</i>	65-120 x 40-60	Oval; the eggs contain 16 to 32 blastomeres	Thin
<i>Chabertia</i>	77-105 x 45-59	Oval; the eggs contain 16 to 32 blastomeres	Thin

Therefore, to aid the identification of different GIN present in mixed infections, flotation-based techniques have to be followed by faecal culture to identify infective third-stage larvae (L3) of GIN. Currently, a number of protocols for coprocultures have been published which differ in temperatures, times and media used for culture and the approach of larval recovery (reviewed in Roeber et al., 2013). In addition, some recent developments have been made towards improving species identification and differentiation of GIN. These include lectin staining for the identification of *H. contortus* eggs (Palmer and McCombe, 1996), computerized image recognition of strongylid eggs (Sommer, 1996), as well as immunological and molecular methods (von Samson-Himmelstjerna et al., 2002; von Samson-Himmelstjerna, 2006). Furthermore, next-generation molecular-diagnostic tools are currently considered a turning point for diagnosis of GIN in small ruminants and other livestock species (Roeber et al., 2013).

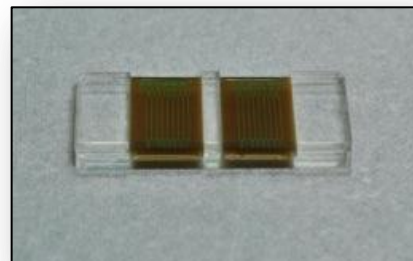
#### 1.2.4. Faecal egg count (FEC) techniques

Copromicroscopic diagnosis of GIN in small ruminants is usually performed by quantitative (FEC) techniques. All FEC techniques are based on the flotation of eggs in an aliquot of faecal suspension from a known volume or mass of a faecal sample (Nicholls and Obendorf, 1994). The results are expressed in terms of eggs per gram of faeces (EPG).

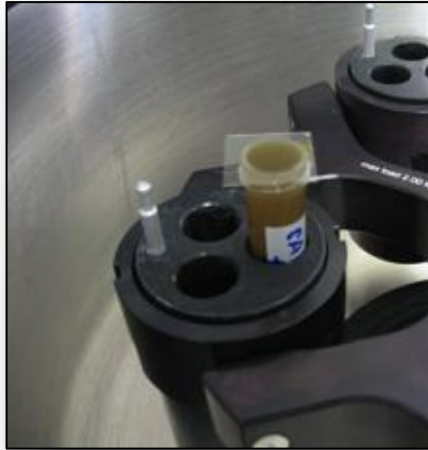
FECs in small ruminants and other livestock species can be performed using different techniques/devices as, for example, McMaster (Fig. 1.3), FECPAK (Fig. 1.4), the flotation in centrifuge (Cornell-Wisconsin technique) (Fig. 1.5), FLOTAC and its derivatives Mini-FLOTAC and Fill-FLOTAC (Fig. 1.6).



**Fig.1.3.** McMaster



**Fig. 1.4.** FECPAK



**Fig. 1.5.** Flotation in centrifuge (Cornell-Wisconsin technique).



**Fig. 1.6.** Devices of the “FLOTAC family”: Fill-FLOTAC, FLOTAC and Mini-FLOTAC.

The McMaster technique, developed and improved at the McMaster laboratory of the University of Sidney (Gordon and Whitlock, 1939; Whitlock, 1948), and whose

name derives from one of the great benefactors in veterinary research in Australia, the McMaster family (Gordon, 1980), is the most universally used technique for estimating the number of helminth eggs in faeces (Rossanigo and Gruner, 1991; Nicholls and Obendorf, 1994). For decades, numerous modifications of this method have been described (Whitlock, 1948; Roberts and O'Sullivan, 1951; Levine et al., 1960; Raynaud, 1970), and most teaching and research institutions apply their own modifications to existing protocols (Kassai, 1999). Many of these modifications make use of different FS, sample dilutions and counting procedures, which achieve varying analytic sensitivities as reported in Figure 1.8 (Cringoli et al., 2004; Roeber et al., 2013). There are at least three variants of the McMaster technique (for details see MAFF, 1986) with different analytic sensitivities: 50 EPG for the “modified McMaster method” and the “modified and further improved McMaster method” or 10 EPG in the case of the “special modification of the McMaster method” (MAFF, 1986).

FECPAK ([www.fecpak.com](http://www.fecpak.com)) is a derivative of McMaster, developed in New Zealand to provide a simple “on farm” method of GIN egg counting for making decisions on the need to treat or to determine whether anthelmintics are effective. It is in essence a larger version of the McMaster slide, having a higher analytic sensitivity (usually 10-30 EPG). The use of such a system requires a significant level of cooperation by farmers and adequate training to ensure that correct diagnoses are made (McCoy et al., 2005).

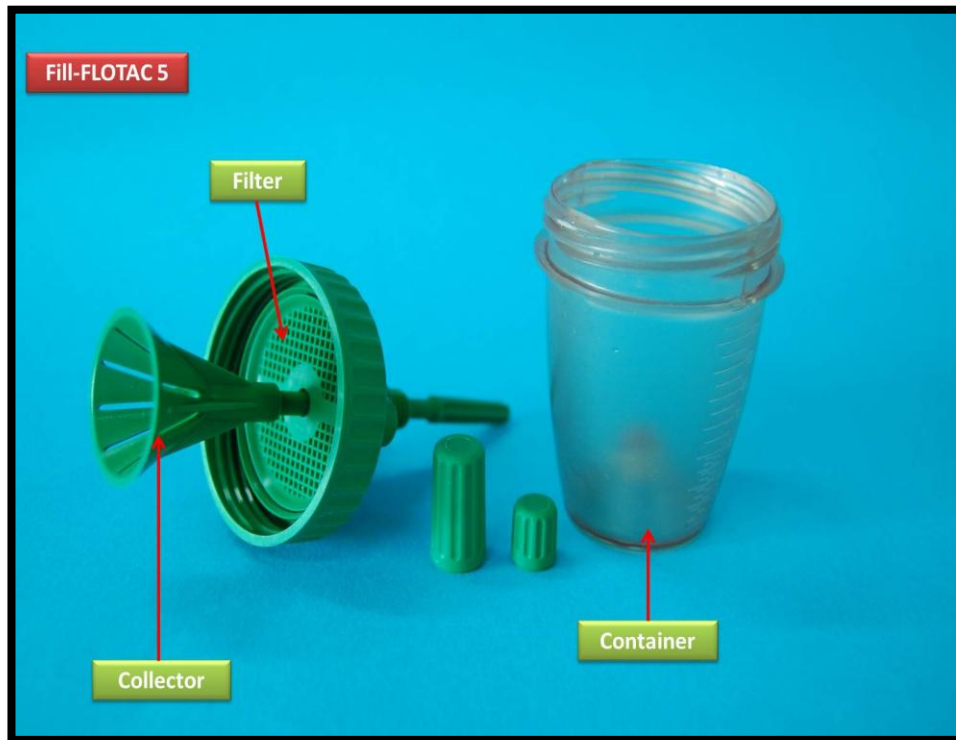
FEC techniques that involve flotation in centrifuge include (Cornell-)Wisconsin (Egwang and Slocombe, 1982) and FLOTAC (Cringoli et al., 2010) both allowing for the detection of GIN up to 1 EPG.

The Wisconsin and modified Cornell-Wisconsin centrifugal flotation techniques (Egwang and Slocombe, 1981, 1982) are highly sensitive methods (analytic sensitivity = 1 EPG or even less depending on the amount of faeces and the dilution factor used) aimed at recovering GIN eggs when in low numbers in bovine faeces. However, they can also be used for FECs of GIN in small ruminants. They are based on flotation in a centrifuge tube and eggs are recovered by means of adding a cover slide to the meniscus of the flotation solution. However, when the number of eggs is high, inefficiencies may arise due to the lack of precision in the egg counting procedures owing to different factors as the possible loosing of some material

during centrifugation, adding the coverslide, and the absence of a grid on the coverslip (Cringoli et al., 2010; Levecke et al., 2012b).

The FLOTAC techniques are based on the centrifugal flotation of a faecal sample suspension and subsequent translation of the apical portion of the floating suspension. The FLOTAC device can be used with three techniques (basic, dual and double), which are variants of a single technique but with different applications. The FLOTAC basic technique (analytic sensitivity = 1 EPG) uses a single FS and the reference units are the two flotation chambers (total volume 10 ml, corresponding to 1 g of faeces). The FLOTAC dual technique (analytic sensitivity = 2 EPG) is based on the use of two different FS that have complementary specific gravities and are used in parallel on the same faecal sample. It is suggested for a wide-ranged copromicroscopic diagnosis (GIN, lungworms, trematoda). With the FLOTAC dual technique, the reference unit is the single flotation chamber (volume 5 ml; corresponding to 0.5 g of faeces). The FLOTAC double technique (analytic sensitivity = 2 EPG) is based on the simultaneous examination of two different faecal samples from two different hosts using a single FLOTAC apparatus. With this technique, the two faecal samples are each assigned to its own single flotation chamber, using the same FS. With the FLOTAC double technique, the reference unit is the single flotation chamber (volume 5 ml; corresponding to 0.5 g of faeces).

A main limitation of FLOTAC is considered the complexity of the technique that involves centrifugation of the sample with a specific device, equipment that is often not available in all laboratories; in addition, studies performed by Levecke et al. (2009) and Speich et al. (2010) demonstrated that FLOTAC is more time consuming than other FEC techniques. To overcome these limitations, under the “FLOTAC strategy” of improving the quality of copromicroscopic diagnosis, a new simplified tool has been developed, i.e. the Mini-FLOTAC, having an analytic sensitivity of 5 EPG (Cringoli et al., 2013). It is a easy-to-use and low cost method, which does not require any expensive equipment or energy source, so to be comfortably used to perform FECs (Cringoli et al., 2013). It is recommendable to combine Mini-FLOTAC with Fill-FLOTAC, a disposable sampling kit, which consists of a container, a collector (2 or 5 gr of faeces) and a filter. Hence, Fill-FLOTAC facilitates the performance of the first four consecutive steps of the Mini-FLOTAC technique, i.e. sample collection and weighing, homogenisation, filtration and filling (Fig. 1.7).



**Fig 1.7.** The main components of Fill-FLOTAC.

The Appendices 3 to 6 of this chapter illustrate the standard operating procedures (SOP) of the FEC techniques mostly used for the diagnosis of GIN in small ruminants, namely McMaster (Appendix 3), Wisconsin (Appendix 4), FLOTAC (Appendix 5) and Mini-FLOTAC (Appendix 6). It should be noted that FEC techniques are considered relatively straightforward and protocols such as the McMaster and the Wisconsin flotation techniques have been available (and remained unchanged) for many years. There is therefore an urgent need of standardizing FEC techniques for an accurate and reliable assessment of GIN intensity and anthelmintic drug efficacy.

#### *1.2.5. Technical variability of FEC techniques*

Each of the FEC techniques described above shows strengths and limitations (Cringoli et al., 2010). Furthermore, they vary considerably according to their performance and operational characteristics (e.g. analytic sensitivity, accuracy and



precision in assessing FECs, timing and ease of use). Figure 1.8 shows the main characteristics (amount of faeces used, reading volume and reading area), analytic sensitivities (multiplication factors when a dilution ratio of 1:10 is used) and timing of the FEC techniques mostly used for the diagnosis of GIN in small ruminants. Therefore, FEC techniques are prone to a considerable technical variability depending also on the selection of the flotation solution, the dilution of the faecal sample, the counting procedure, the reading area and many other factors reported in the following sections.

Furthermore, other important technical factors that affect FECs include:

- (i) variability arising from the quantity of faeces excreted by the animals. Where precise measurements of faecal egg output are required the total daily egg output should ideally be determined by collecting and weighing all the faeces passed in a 24-hour period (MAFF et al., 1986; Cringoli et al., 2010).
- (ii) variability arising from the fact that the parasite eggs are not evenly distributed through the faeces. Homogenization of fecal material has been suggested as one way to overcome intra-specimen variation of FECs (Cringoli et al., 2010; Mekonnen et al., 2013). However, the effect of homogenization on helminth FECs has yet to be determined.
- (iii) variability arising from a possible diurnal fluctuation in FECs. Indeed, parasites egg excretion in faeces may be subjected to hour-to-hour and/or day-to-day variation due to endogenous or exogenous factors (Villanua et al., 2006). However, studies regarding the possible hour-to-hour and day-to-day fluctuation of GIN eggs in small ruminants have not been performed so far.
- (iv) variability arising from the storage of the faecal sample. This factor is of great importance because, if not performed appropriately, it can cause a significant artefactual reduction in GIN egg numbers primarily due to

hatching of eggs or biological degradation (Nielsen et al., 2010). To circumvent this problem, different strategies, such as refrigeration (Nielsen et al., 2010; McKenna, 1998) and chemical preservation (Whitlock, 1943; Foreyt, 1986, 2001) have been suggested. Some general recommendations are often given to keep GIN eggs as fresh and undeveloped as possible (for up to 7 days). These include keeping faeces at 4°C (Le Jambre, 1976; Smith-Buys and Borgsteede, 1986) or in airtight containers to produce an anaerobic environment (Hunt and Taylor, 1989). It should be noted that, if nematode larvae are to be cultured for identification, samples should not be stored at 4-8°C for more than 24 h as this may affect the hatching of eggs of *H. contortus* and *Cooperia* (McKenna, 1998). Chemical preservation can also be used but limitations must be underlined. As an example, in a study by Foreyt (1986), storage by either freezing or using formalin (10%), ethyl alcohol (70%) or methyl alcohol (100%) was very inefficient for recovery of nematode eggs (primarily *Haemonchus* and *Ostertagia*) in deer faecal samples. Similarly, van Wyk and van Wyk (2002) demonstrated that freezing of sheep faeces invalidated *Haemonchus* FECs by the McMaster technique and suggested that FECs from cryopreserved faeces (whether in a freezer at -10 °C or in liquid nitrogen) should be regarded as being inaccurate (van Wyk and van Wyk, 2002).

<b>FEC technique</b> (amount of gaeces used)	<b>Volume</b> (ml)	<b>Reading</b> <b>Area</b>	<b>Analytic</b> <b>Sensitivity</b>	<b>Timing</b>
<b>McMASTER</b> (3 to 5 g)	0.15 ml	100 mm <sup>3</sup>	66.6	4 min
	0.30 ml	200 mm <sup>3</sup>	33.3	(Levecke et al., 2009)
	0.50 ml	324 mm <sup>3</sup>	20	
	1 ml	648 mm <sup>3</sup>	10	
<b>FecPak</b> (10 g)	0.5 ml	216 mm <sup>3</sup>	20	Less than 10 min
	1.0 ml	432 mm <sup>3</sup>	10	(www.techiongroup.co.nz)
	1.4 ml	546 mm <sup>3</sup>	7.1	
	2.8 ml	1092 mm <sup>3</sup>	3.6	
<b>Cornell- Wisconsin</b> (3-5 g)	15 ml	324 mm <sup>3</sup>	1	15-20 min (Egwang and Slocombe 1992)
<b>FLOTAC</b> (10 g)	10 ml	648 mm <sup>3</sup>	1	12-15 min (Cringoli et al., 2010)
<b>Mini-FLOTAC</b> (5 g)	2 ml	648 mm <sup>3</sup>	5	10-12 min (Barda et al., 2013)

**Fig. 1.8.** Schematic features (amount of faeces, reading volume, reading area, analytic sensitivity at 1:10 dilution ratio and timing) of McMaster, FECPAK, Cornell-Wisconsin, FLOTAC and Mini-FLOTAC techniques.

### 1.3. PHYSICAL, BIOLOGICAL AND EPIDEMIOLOGICAL FACTORS AFFECTING FECs OF GIN IN SMALL RUMINANTS

A part from the operational and performance characteristics of the FEC techniques and the sources of technical variability described in the previous section, FEC results will depend on a plethora of different factors, including:

- (i) physical parameters such as, for example, consistency (water content) of faeces; and
- (ii) biological/epidemiological parameters related either to the parasite, the host and the environment such as, for example, fecundity of worms, season of sampling, age and sex of animals, and immunity development.

#### 1.3.1. Consistency of faeces

Samples intended for faecal analysis can be of varying consistencies, being soft to watery (diarrhoeic) or hard and desiccated (mostly from animals following transport and without access to food or water) (Gordon, 1953, 1981). A series of correction factors have been recommended to correct for the dilution effect on FECs in sheep. Gordon (1967) suggested the following categories of faecal consistency and correction factors (multipliers): pellets = 1; soft formed = 1.5; soft = 2; very soft = 2.5 and diarrhoeic = 3–3.5. Recently, a new adjustment factor based on the prediction of dry matter from a faecal consistency score (FCS) has been proposed by Le Jambre et al. (2007) using the following formula: adjustment factor =  $1 + (\text{FCS} - 1/2)$ . FCS is classified on the following scale: 1 = normal formed pellets; 1.5 = pellets losing their form; 2 = faeces have no pellet form; 3 = faeces wet but do not run on a flat surface; 4 = watery faeces that run on a flat surface but maintain a depth >2 mm; 5 = watery faeces that run on a flat surface and do not maintain a depth >2 mm (Le Jambre et al., 2007).

#### 1.3.2. Fecundity of female worms

The biotic potential of different species of GIN varies (Gordon, 1981) and parasite density and immune mediated “control” by the host have been shown to influence the egg production (fecundity) of female worms in different species (Rowe et al., 2008; Stear and Bishop, 1999). Indeed, some GIN species as *H. contortus* and *Oesophagostomum venulosum* are known to be highly fecund species (Robert and Swan, 1981, 1982; Coyen et al., 1991), whereas some others show a low fecundity,

such as species of *Teladorsagia (Ostertagia)* (Martin et al., 1985), *Trichostrongylus* (Sangster et al., 1979) and *Nematodirus* (Martin et al., 1985; McKenna, 1981). As an example, a field study by Coyen et al. (1991) on the fecundity of GIN of naturally infected sheep showed the following estimated average fecundities (eggs/female/day): *H. contortus* (6,582); *Trichostrongylus* spp. (262); *Nematodirus* spp. (40); and *O. venulosum* (11,098). Another study conducted by Stear and Bishop (1999) demonstrated that fecundity of *T. circumcincta* was skewed and ranged from 0 to 350 eggs/female/day.

### 1.3.3. Relation between FECs and worm burden

There is no agreement in the literature to establish whether FECs are correlated to worm burden and may predict the intensity of GIN infection.

The relation between FECs and worm burden will depend on various factors related to the host, the parasite and the environment. For example, FECs for adult cattle do not usually correlate with worm burden (McKenna, 1981). In small ruminants infected with *H. contortus* (Roberts and Swan, 1981; Coadwell and Ward, 1982) or *T. colubriformis* (Beriajaya and Copeman, 2006) FECs are strongly correlated with worm burden. However, this relationship does not hold true for infection with *Nematodirus* spp. (Cole, 1986) and *T. circumcincta* (Jackson and Christie, 1979). In addition, in areas where co-infection with many nematode species occurs, the relatively high egg production of *H. contortus* may tend to mask the much lower egg production of species such as *T. colubriformis* and *T. circumcincta* (Roeber et al., 2013). The relation between FECs and worm burden could be also influenced by factors related to the host (e.g. age and immunity development). As an example, McKenna (1981) showed a correlation coefficient of 0.74 between FECs and worm counts (*Nematodirus* excluded) in young sheep (up to 12 months of age); in contrast in “old” sheep (over 12 months of age) the corresponding correlation coefficient was 0.23. Therefore, as a consequence of the effect of age and development of host immunity on reduction in egg laying, there could be no relationship between worm burden and GIN egg counts. So whilst FECs may give an indication of worm burdens in young animals this does no longer applies in older animals, unless the host species develops little or no natural immunity (McKenna, 1981, 1987).

Another important issue to mention is the importance of the GIN hypobiotic larval populations upon the relationship between total worm burden and FEC. Indeed, it is well known that, under unfavourable conditions, the GIN larvae undergo a period of hypobiosis (arrested development; typical for species of *Haemonchus* and *Teladorsagia*). Hypobiotic larvae usually resume their activity and development in spring in the case of *Haemonchus* or autumn in the case of *Teladorsagia*. This may be synchronous with the start of the lambing season, manifesting itself in a periparturient increase in FECs in ewes (Salisbury and Arundel, 1970).

#### *1.3.4. Overdispersion of GIN egg counts*

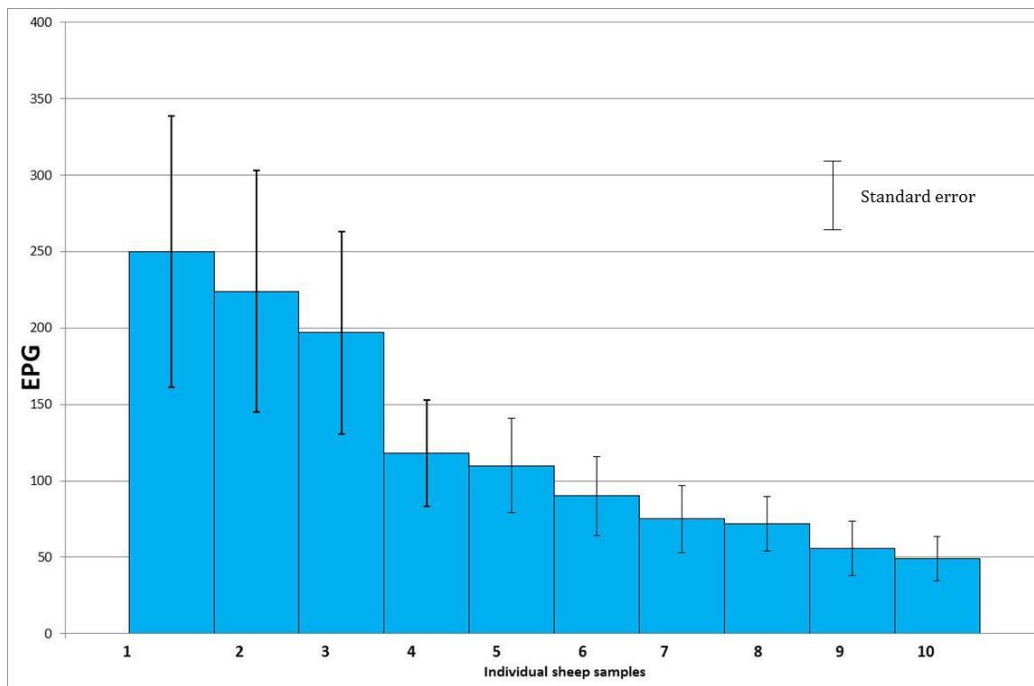
The distribution of egg counts and parasites between different animals within a group is well known to be overdispersed (Shaw and Dobson, 1995; Grenfell et al., 1995; Wilson et al., 1996; Shaw et al., 1998; Morgan et al., 2005; Torgerson et al., 2005, 2012). The non-random distribution of eggs within a faecal sample will conform to a Poisson process and thus repeated calculations of EPG from the same faecal sample will be subject to Poisson errors (Torgerson et al., 2012). Therefore there is inevitable variability in evaluating FECs even with a highly precise laboratory technique due to this random variation. This is partly due to dilution or detection limits (i.e. analytic sensitivity) of the FEC techniques magnifying Poisson errors and, importantly, due to aggregation of parasite infection between hosts (Torgerson et al., 2014). The overdispersed distribution of egg counts can be modelled with the negative binomial distribution (Torgerson et al., 2005) or other skewed or zero inflated distributions (Torgerson et al., 2014).

Overdispersion presents a serious risk of bias, since the mean of a small subsample of individual FECs is very likely to underestimate the group mean FECs (Gregory and Woolhouse, 1993), leading to misguided advice and potentially erroneous treatment decisions. Overdispersion also complicates comparisons between mean FECs, e.g. in tests for anthelmintic resistance (Cabaret and Berrag, 2004; Morgan et al., 2005; Torgerson et al., 2005).

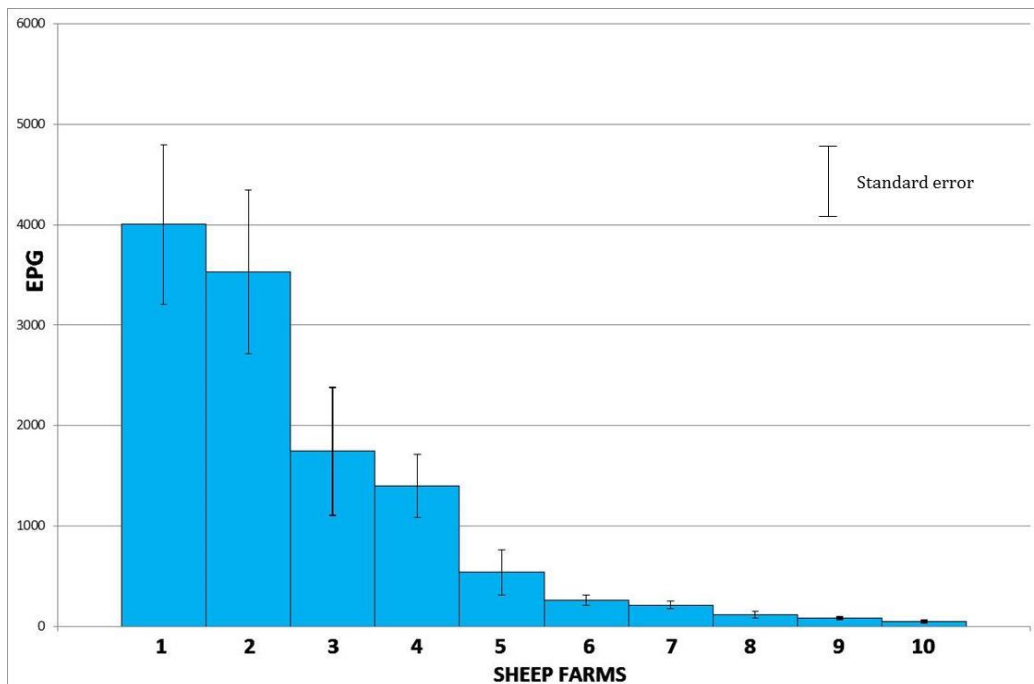
Examples of variability of GIN egg counts (EPG) among different individual sheep within a farm (intra-farms) and among different farms (inter-farms) are given in Figures 1.9 and 1.10, respectively.

It should be noted, however, that variability of GIN egg counts (EPG) among

different farms (Fig. 1.10) is likely due to multiple factors (e.g. management, treatments, etc.) and not only on biological/epidemiological issues.



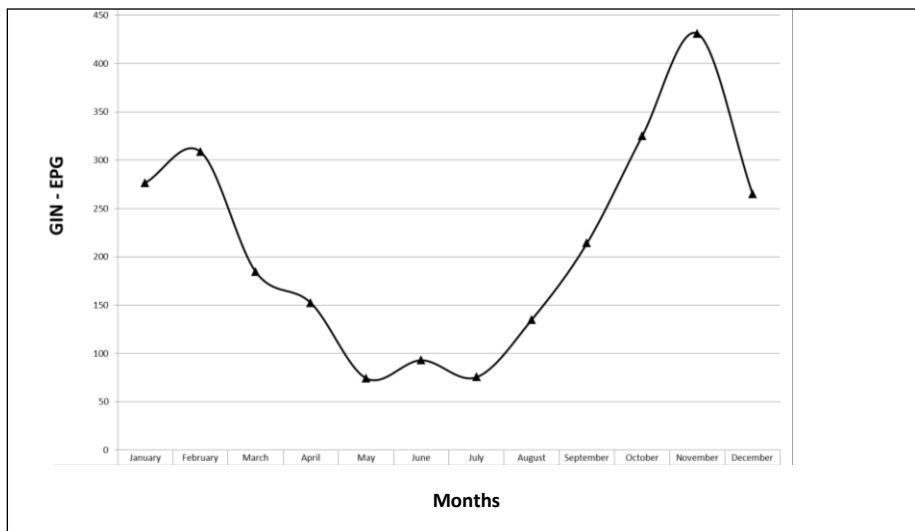
**Fig. 1.9.** Variability of GIN egg counts (mean EPG and standard errors) among different individual animals sampled in a sheep farm in southern Italy (unpublished data).



**Fig. 1.10.** Variability of GIN egg counts (mean EPG and standard errors) among different sheep farms sampled in southern Italy (unpublished data).

### 1.3.5. Seasonal variations

The seasonal patterns of GIN infection in small ruminants should be also considered as factor affecting FECs, in order to select the best period (months) of conducting helminth egg counts. GIN egg counts are strongly influenced by the period of sampling (seasonality) and will vary greatly from one month to the next, one year to the next and between geographical locations depending on the prevailing climatic and environmental conditions but also on the management practices (Cringoli et al., 2008; Morgan et al., 2013). Figure 1.11 shows a typical seasonal pattern of GIN egg counts in sheep in southern Italy (a region with a Mediterranean climate) with two peaks of EPG (February and November) and a ditch (May to June).



**Fig.1.11.** GIN egg count pattern in sheep in southern Italy.

Similarly, Doligalska et al. (1997) showed that FEC variation is usually continuous but heavily skewed in sheep in Poland where the mean and variance of FECs differ within seasons and years of sampling (Doligalska et al., 1997). McMahon et al. (2013), in studies performed in Northern Ireland, showed that pasture contamination levels of GIN are at their highest over the period September-October having increased steadily over the immediately preceding months (March-May) (McMahon et al., 2013). Other similar studies performed in Canada, demonstrated that GIN peaks occur in spring for the ewes and in summer for the lambs (Mederos et al., 2010).



### *1.3.6. Host and parasite factors*

Other important factors affecting FECs in small ruminants include the age, sex and physiological status of the animals. As an example, it is well known that high GIN egg production is usually observed in ewes during the periparturient period (PP). The so-called peri-parturient rise (PPR) is a major source of GIN pasture contamination for both lambs and ewes (Barger, 1999). Dunsmore (1965) suggested that both environmental and physiological factors might be important contributors to the PPR. Some authors believe the PPR is linked to the ewes' productivity stage, and the endocrine, immunological, and metabolic changes that ensue (Taylor, 1935; Crofton, 1954; Brunsdon, 1970; Michel, 1976; Jeffcoate and Holmes, 1990; Coop and Holmes, 1996; Donaldson et al., 1998; Beasley et al., 2010). Beasley et al. (2010) showed that changes consistent with a reduction in immunity expression occurred in both pregnant and lactating ewes. These changes in immunity may facilitate the parasites' establishment within the host, enhance their prolificacy, and increase their longevity (Michel, 1976). It is a commonly expressed viewpoint that PPR most likely eventuates from complex interactions between the endocrine and immune systems; however, these interactions may be, in turn, influenced by the nutritional environment and metabolic status of the periparturient ewes. In the study by Beasley et al. (2010), the mobilization of fat and protein reserves, indicative of an underlying nutrient deficit throughout lactation in suckled ewes, and closely associated leptin and cortisol profiles, provided strong evidence of an underlying nutritional basis for the PPR.

Additional considerations regarding the host-parasite relationship are that FECs (i) only reflect patent but not pre-patent infections (Thienpont et al., 1986), (ii) do not provide any information regarding male or immature worms present (McKenna, 1981) and (iii) can be influenced by variation in times of egg excretion by adult worms (Villanua et al., 2006) and age of the worm population (Thienpont et al., 1986).

## **1.4. THE USE (INTERPRETATION) OF GIN EGG COUNTS IN SMALL RUMINANTS**

The use (interpretation) of FECs is of great relevance in small ruminant farming in

order to:

- ✓ estimate intensity of GIN infections on a farm ;
- ✓ assess need for control (therapeutic or chemoprophylactic);
- ✓ predict levels of pasture contamination;
- ✓ determine efficacy of anthelmintics and long-term control programme.

FECs have long been used in farm animal veterinary practice to estimate intensity of GIN infections. However, problems arise regarding the number of animals to test and frequency of sampling for a FEC being informative to estimate intensity of GIN infections at farm level and predict levels of pasture contamination (Sargison, 2013). In small ruminants, GIN egg counts are generally performed on samples taken from 10/20 animals within a group, and usually show standard deviations that are similar to the arithmetic mean values. Thus, the individual FECs of animals within groups with a mean FEC of 450 EPG might be 50 or 1000 EPG, neither of which provides valid information about the level of challenge to the individual or to the group or about the need for anthelmintic drug treatment (Sargison, 2013). Monitoring FEC has been suggested to optimize “flock parasitological managing”. However, given the wide regional variation that exists between sheep management systems and the different parasites that inhabit them, there are no universally applicable “blueprint” approaches to monitoring FECs for the control of GIN infections at farm level (Jackson et al., 2009). Therefore, besides FEC, accumulated experience of local epidemiological patterns, as well as knowledge of pastures and grazing history, should be regarded as extremely valuable information to estimate intensity of GIN infections on a farm and assess need for control (Charlier et al., 2014). Another area in which FECs can also provide useful information is to indicate levels of pasture contamination, triggering group treatment to reduce the infection pressure, together with good practices of pasture management; however, this approach is yet to be widely and systematically used in practice (Charlier et al., 2014).

Anthelmintic drugs are commonly used in sheep farms either for prophylactic purposes, in which the timing of treatment is based on knowledge of the epidemiology, or for therapeutic purposes to treat existing infections or clinical outbreaks (Getachew et al., 2007). FEC is often used as indication of flock-scale parasitism as the basis for drenching. This usually entails periodically taking faecal

samples for worm egg counts, and treating when counts exceed a “trigger level” associated with parasitism (Besier, 2012). However, rigid interpretation of FEC results can be potentially misleading (Sargison, 2013). Indeed, not only there are no widely accepted defined FEC thresholds for treatment decisions, and thresholds will vary in function of the nematode species that is involved (Charlier et al., 2014). Some authors suggest that less than 500 EPG is considered a low level of GIN infection, between 500 and 1500 EPG as moderate to high, and more than 1500 EPG as high level of infection (Hansen and Perry, 1994). According to other authors FEC of  $\geq 200$  EPG is regarded to indicate a significant worm burden and is used as basis for the decision for anthelmintic treatment ([www.wormboss.com.au](http://www.wormboss.com.au)). Other authors suggest a threshold of 300-500 EPG (based on counts of 10 animals) for treatment of sheep flocks (Coles G.C., personal communication). It is therefore clear that there is a misleading view of FEC thresholds for treatment in sheep and longitudinal studies justifying these values are lacking. Also, there is no established threshold even for worm burden. Therefore, to gain maximal information from FECs, strict thresholds for treatment should not be applied, instead baseline FEC data (i.e. longitudinal data) should be established so that it can be determined when EPGs deviate for what can be expected on a particular farm.

Furthermore, FECs have long been used to determine efficacy of anthelmintics and control programmes in livestock. The faecal egg count reduction test (FECRT), with its ability to provide a measure of the performance of a number of different anthelmintics at a time, is one of the most widely used methods for on-farm assessment of anthelmintic efficacy (McKenna, 2002, 2013). The FECRT is simple and relatively easy to perform (Demeler et al., 2012). Guidelines for the performance of a FECRT have been published (Coles et al., 1992) and reviewed (Coles et al., 2006) but they should be updated. Indeed, the data obtained by FECRT have been reported not to be highly reproducible (Miller et al., 2006) and a straightforward interpretation is hindered by a number of limiting factors associated with the FECRT (Levecke et al., 2012a,b). Factors unrelated to treatment, such as non-uniform distribution of eggs in the faeces and inappropriate drug administration, can further complicate the interpretation of FECRT data (Roeber et al., 2013). The following Table 1.3 (adapted from Roeber et al., 2013) summarizes the main principles and limitation of FECRT.

**Table 1.3.** Summary of principles and limitations of FECRT (adapted from Roeber et al., 2013).

<b>Assay</b>	<b>Principle</b>	<b>Comments and existing limitations</b>
Faecal egg count reduction test	Provides an estimate of anthelmintic – efficacy by comparing faecal egg counts from sheep before and after treatment. Resistance is declared if reduction in the number of eggs counted is <95% and the lower confidence interval for the percentage of reduction is below 90%.	<ul style="list-style-type: none"><li>- Does not accurately estimate the efficacy of an anthelmintic to remove worms.</li><li>- It rather measures the effects on egg production by mature female worms.</li><li>- Different anthelmintics require sample collection at different time intervals.</li><li>- No agreed standard for FEC method or for the calculation of reduction.</li><li>- Results can be inconclusive due to low analytical sensitivity of the technique.</li><li>- Different results in repeated experiments.</li><li>- Does not provide species specific information if undifferentiated. Larval culture required for further differentiation.</li></ul>

Another area in which FECs can also provide useful information is to evaluate the benefits of control programs. Long-term monitoring FECs and FECR on sheep farm could potentially play an important role as indicators for anthelmintic treatment decisions in optimised helminth control strategies such as targeted treatment (TT) or targeted selective treatment (TST). In particular FEC may offer benefits as it can allow treatments to be adapted to seasonal and temporal changes in GIN prevalence (Charlier et al., 2014).

## **1.5. CONCLUSIONS AND RESEARCH GAPS**

Although widely used in veterinary parasitology, FEC/FECR techniques are prone to a number of shortcomings.

*First*, there is a clear lack of standardization of FEC techniques and usually each lab uses “its own” method mostly based on the “lab traditions” rather than on the performance (e.g. sensitivity, specificity, reproducibility, negative predictive value), or operational characteristics (e.g. simplicity, ease of use, user acceptability) of the technique (Rinaldi and Cringoli, 2014). However, FEC techniques are subjected to technical variability due to faecal storage before analyses, the amount of faeces under analysis, the homogenization of faecal sample, the selection of the FS, the FEC technique and counting procedure used, and many other factors. In addition, several physical, biological (host-parasite-related) and environmental factors strongly affect FECs of GIN and therefore these factors should be taken into consideration when interpreting FEC results in small ruminants as in other livestock species. All these aspects have been poorly investigated so far and new research is needed on this topic.

*Second*, the results of any copromicroscopic technique strongly depend on the accuracy of laboratory procedures but also on the experience of the laboratory technicians reading the microscopic fields (Utzinger et al., 2012). Therefore, the “human” factor (i.e. the hands and eyes of technicians) is of fundamental importance for copromicroscopic analyses compared to other diagnostic approaches (i.e. immunological or molecular methods). However, there is often a lack of inter-laboratory standardization of FEC techniques, as well as an absence of internal and external quality control for parasitological diagnosis.

*Third*, the main limitation of copromicroscopy is the time and cost to conduct FECs on a representative number of animals and alternative approaches are therefore needed. A potentially useful alternative to reduce the workload is to examine pooled faecal samples, in which equal amounts of faeces from several animals are mixed together and a single FEC is used as an index of group mean FECs (Morgan et al., 2005). However, there are still many issues to be clarified and standardized before the pooled FEC can be introduced in the routine diagnosis of GIN and, by extension, in the assessment of anthelmintic drug efficacy (FECR) in ruminant farms. These include, for example, the effect of pool size (i.e. the number of individual samples in each pool) as well as the effect of analytic sensitivity of the FEC technique used.

In conclusion, this literature review identified several research gaps regarding the variability, use, interpretation and limitations of FEC/FECR techniques in small ruminants. The lack of detailed and up-to-date studies on this topic, justify the specific objectives of this thesis towards the challenge of bringing together parasitological research and veterinary practice for the achievement of advances in small ruminant farming in Europe and beyond.

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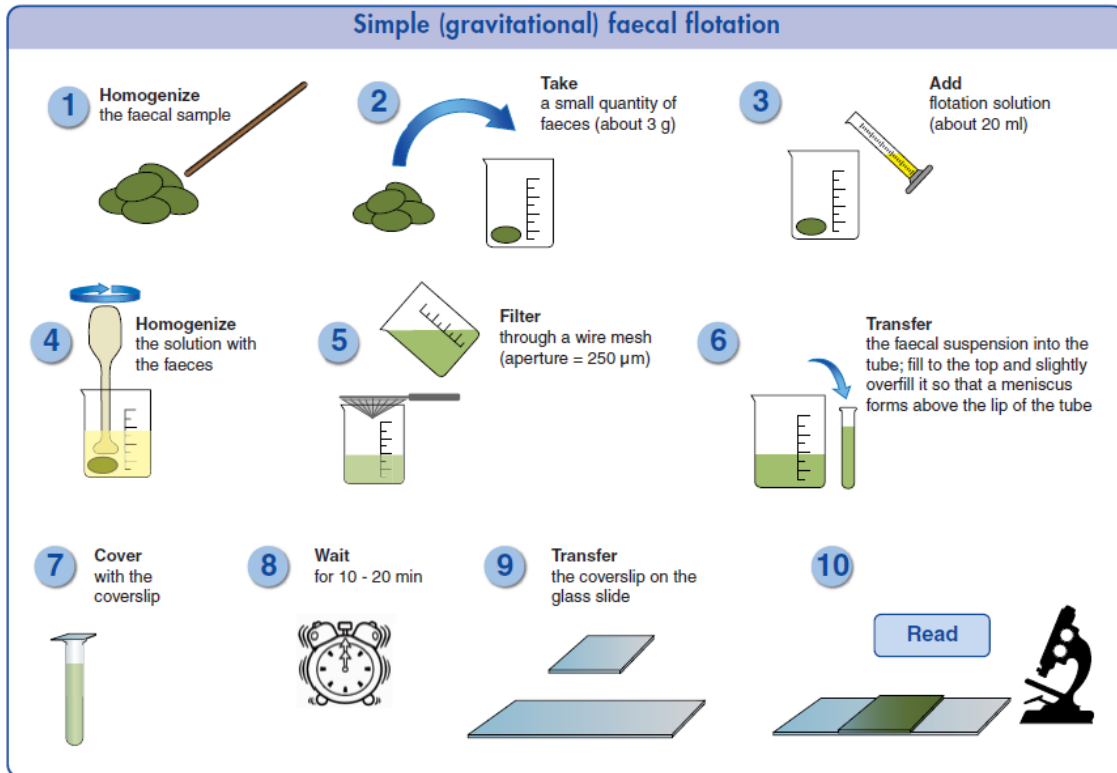
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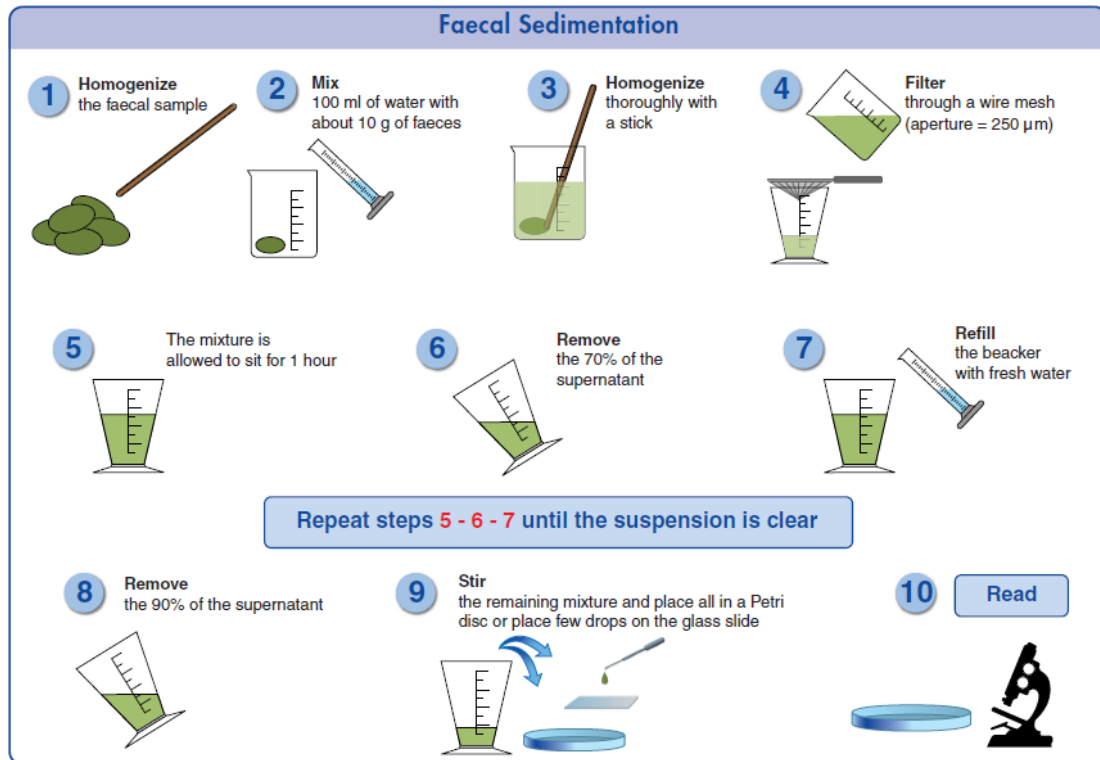
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## 1.7 APPENDICES

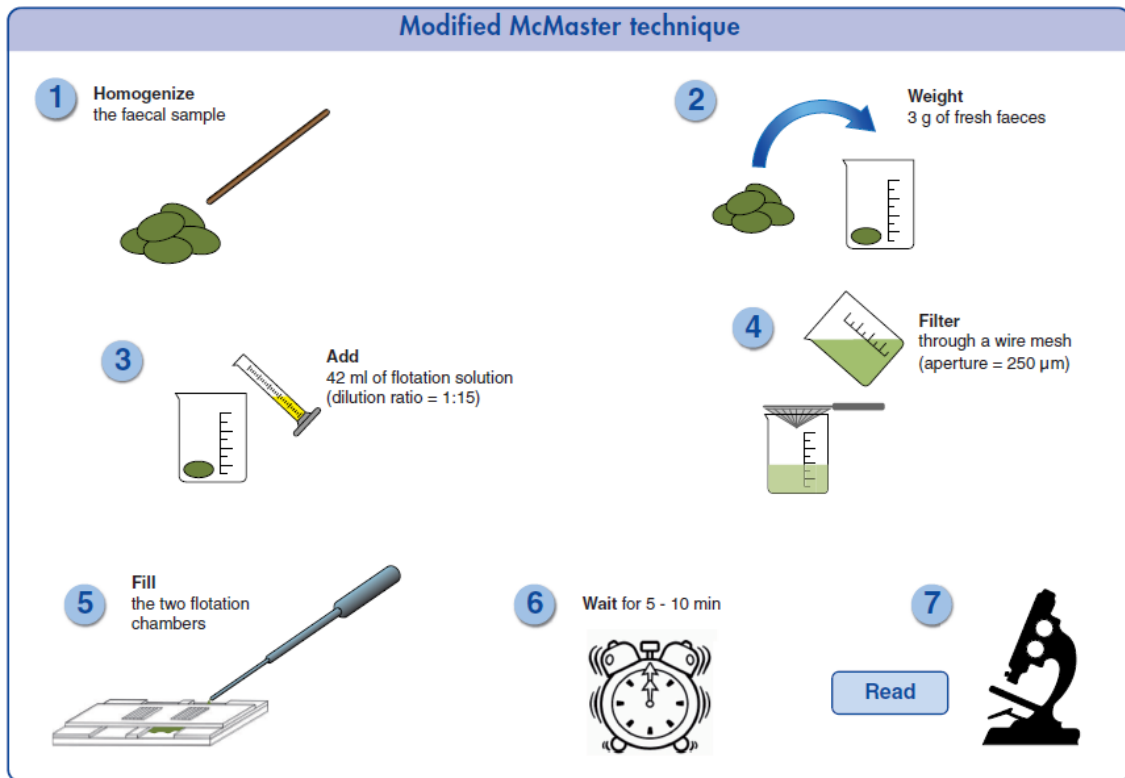
### APPENDIX 1



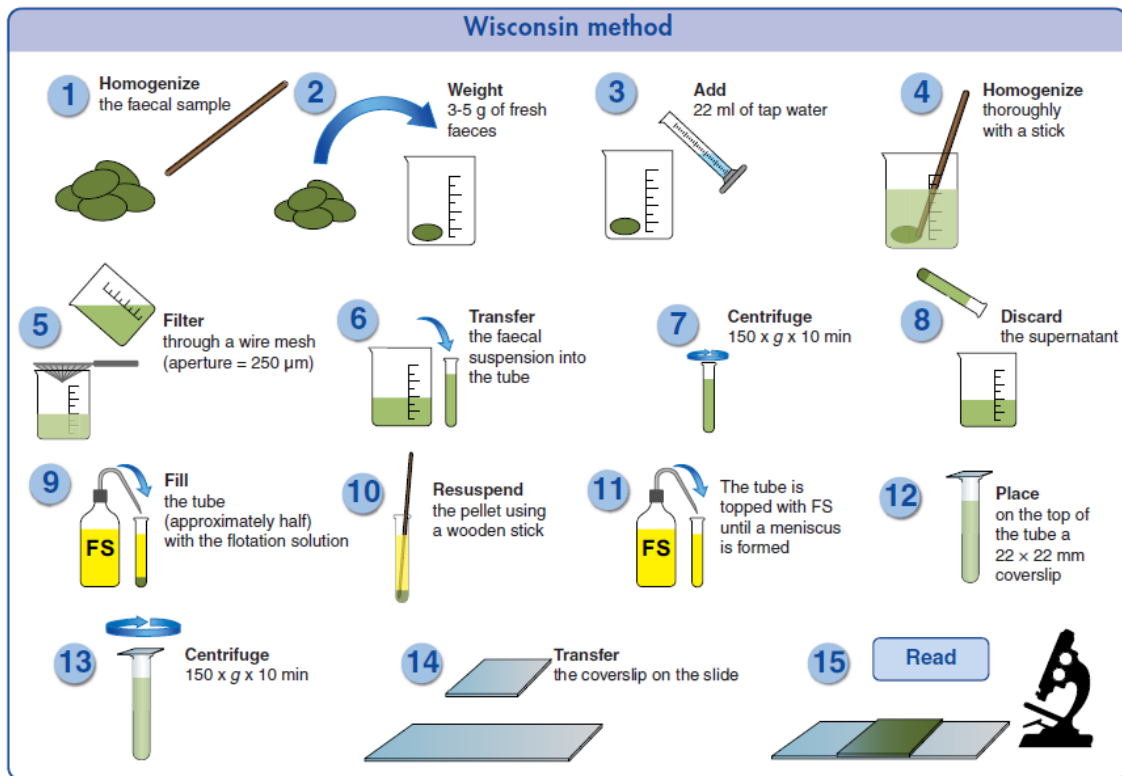
## APPENDIX 2



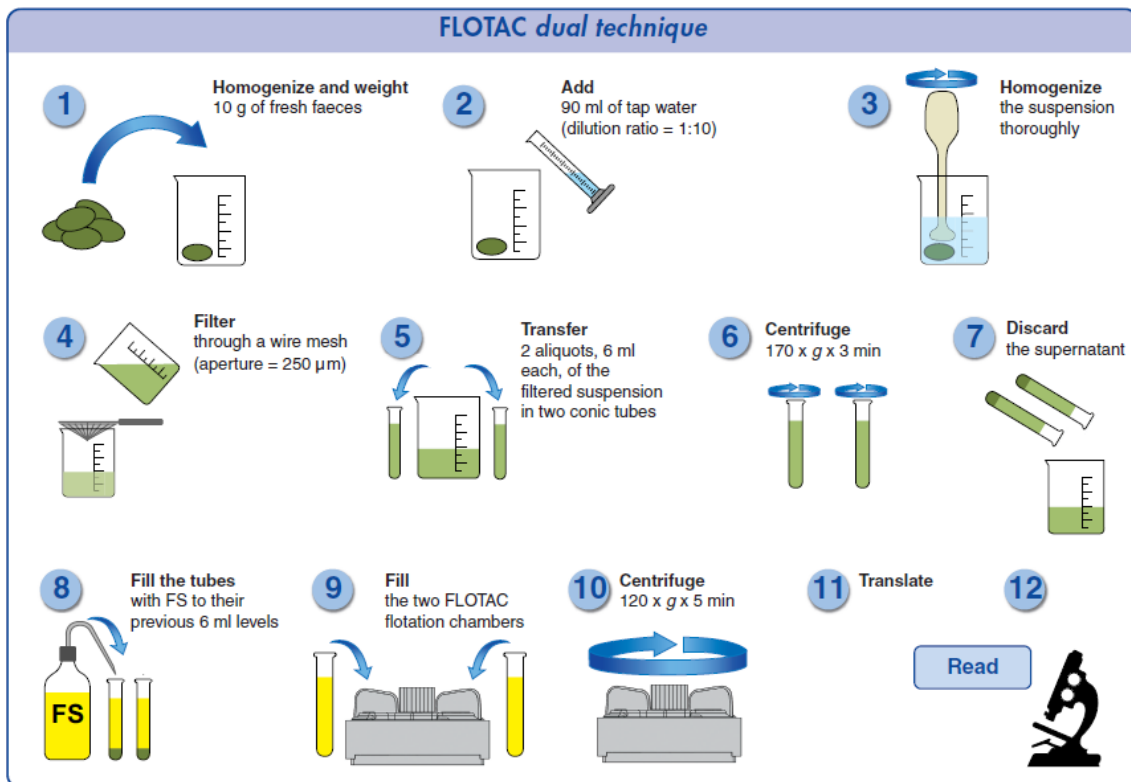
## APPENDIX 3



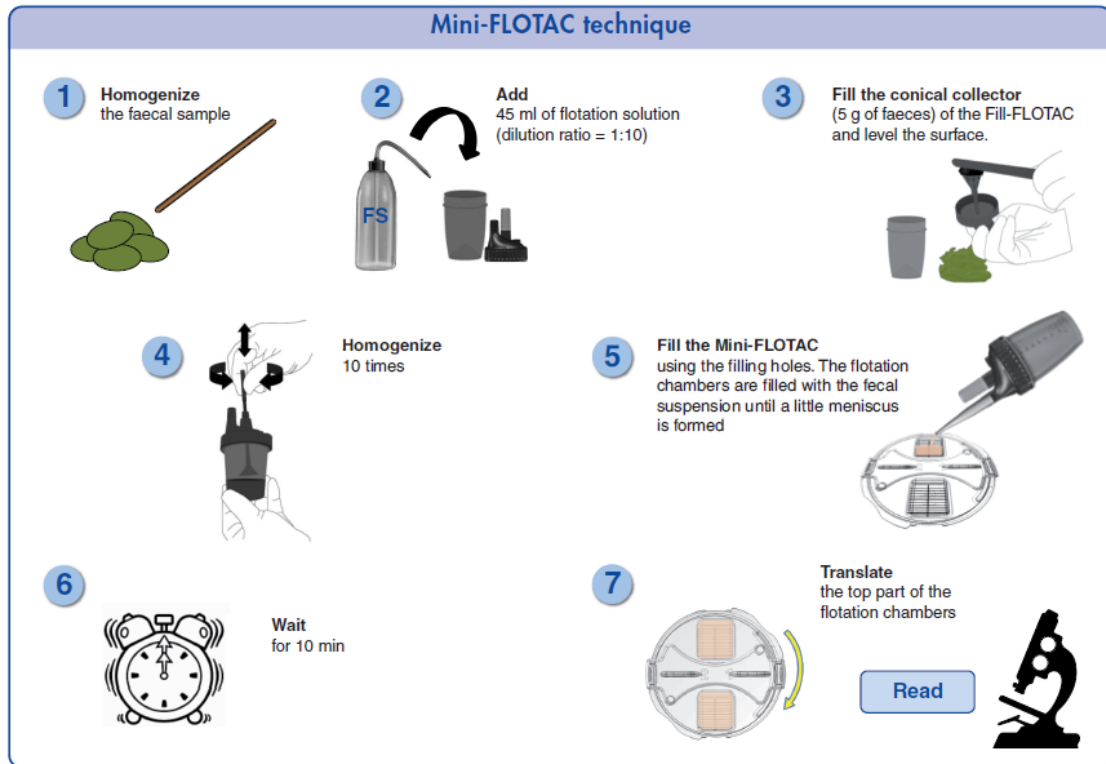
## APPENDIX 4



## APPENDIX 5



## APPENDIX 6





## OBJECTIVES

The overall aim of the thesis was to study the different aspects concerning the coprological diagnosis of gastrointestinal nematode (GIN) infections in small ruminants with particular emphasis on the significance, interpretation and limitations of faecal egg count (FEC). Particular attention was given to the introduction of a new tools, Mini-FLOTAC and Fill-FLOTAC.

The specific objectives were:

1. To determine the value of pooled faecal samples to assess GIN infection intensity (FEC) and anthelmintic efficacy (FECR). For this purpose, field trials were conducted to: (i) compare FEC and FECR from individual sheep samples and pools of different size (5, 10 and 20 individual sheep samples); (ii) assess the effect of three different analytic sensitivities (10, 15 and 50) on individual and pooled samples using McMaster (analytic sensitivities = 15 and 50) and Mini-FLOTAC (analytic sensitivity = 10) and; (iii) determine the effect of the pooling on FECR [Chapter 2 ].
2. To define the accuracy of the Mini-FLOTAC technique (using Mini-FLOTAC and Fill-FLOTAC) and to compare its performance and operational characteristics with those of other FEC techniques. For this purpose, laboratory trials were conducted on sheep faecal samples to calibrate the Mini-FLOTAC compared to simple flotation, Conrell-Wisconsin, FECPAK and McMaster techniques. A further aim was to identify the best flotation solution (FS) and to evaluate the influence of faecal preservation methods combined with FS on GIN egg counts [Chapter 3-4].
3. To discuss the present assessments and future perspectives of FEC/FECR in small ruminants, and implications for epidemiological investigations on GIN infections and for use in control programmes [Chapter 5].

## CHAPTER 2

### *Comparison of individual and pooled faecal samples in sheep for the assessment of gastro-intestinal nematode infection intensity and anthelmintic drug efficacy using McMaster and Mini-FLOTAC\**

\*Based on the manuscript: Rinaldi, L., Levecke, B., Bosco, A., **Ianniello, D.**, Pepe, P., Charlier, J., Cringoli, G., Vercruyse, J. Comparison of individual and pooled faecal samples in sheep for the assessment of gastro-intestinal strongyle infection intensity and anthelmintic drug efficacy using McMaster and Mini-FLOTAC. *Vet. Parasitol.*, 2014. 15, 216-223.

## 2.1 INTRODUCTION

The specific and sensitive diagnosis of gastro-intestinal nematode (GIN) infections of livestock underpins effective disease control, which is now particularly important given the problems associated with anthelmintic resistance (AR) in parasite populations (Morgan et al., 2013; Roeber et al., 2013a,b). Currently, diagnosis of these infections relies predominantly on copromicroscopy (Cringoli et al., 2010) and faecal egg count (FEC) techniques are the most widely used methods to estimate GIN intensity through the assessment of eggs per gram of faeces (EPG). Moreover, reduction in faecal egg count (FECR) is the method of choice to monitor anthelmintic drug efficacy and to detect AR in ruminants (Coles et al., 1992, 2006).

However, there are still some obvious limitations that will affect the use of FEC/FECR. From a general point of view, the main limitation of copromicroscopy is the time and cost to conduct FECs on a representative number of individual animals. An alternative to reduce the workload is to examine pooled (composite) faecal samples, in which equal amounts of faeces from several animals are mixed together and a single FEC is used as an index of group mean FECs. In their simulation-based study, Morgan et al. (2005) suggested that GIN egg density in a well-mixed composite sample from 10 sheep (3 g of faeces from each), estimated by examination of four McMaster chambers, is likely to provide an adequate estimate of group mean FEC in the majority of situations.

Similarly, examination of pooled samples in field studies was shown as a quick and valid alternative to the examination of individual samples for monitoring GIN infections by means of FECs in sheep and cattle in Australia (Baldock et al., 1990; Ward et al., 1997). Some other studies have described the use of pooled FECs for assessing infections by helminths (not only GIN) in sheep for farm routines and in cross-sectional prevalence surveys (Nicholls and Obendorf, 1994; Cringoli et al., 2002; Musella et al., 2011).

However, there are still many issues to be clarified before the pooled FEC is introduced in the routine diagnosis of GIN and, by extension, in the assessment of anthelmintic drug efficacy (FECR) in ruminant farms.

First, the effect of pool size (i.e. the number of individual samples in each pool) has not been estimated in sheep so far and arbitrary numbers of individual faecal

samples were used, ranging from 3 (Baldock et al., 1990) to 10 (Morgan et al., 1995). However, the effect of pool size has been already investigated in goats, for GIN (Cabaret et al., 1986) and coccidian (Chartier, 1991).

Second, the effect of analytic sensitivity of the FEC technique on pooling has not been evaluated so far and the McMaster technique (MAFF, 1986) was usually employed with an analytic sensitivity of 15 or 50 eggs per gram (EPG) of faeces. It is likely that a FEC technique with a higher analytic sensitivity might be used to pool a greater number of samples. The recently developed Mini-FLOTAC (Cringoli et al., 2013) (Fig. 2.1) having an analytic sensitivity of 10 EPG may provide an alternative to the commonly applied McMaster for quantitative copromicroscopy in ruminants (Da Silva et al., 2013) in order to perform FECs on pooled samples. Also, the effect of mixing (homogenization) procedure has not been evaluated so far.



**Fig. 2.1.** Mini-FLOTAC

Third, there is little information on the application of pooled FECs to decide on control programmes and in drug efficacy studies to assess FECR. In their recent simulation study, Calvete and Uriarte (2013) reported that pooling samples is one interesting option for FECR tests since it considerably reduces the workload.

In order to clarify some of these three key aspects concerning the effect of pooling faeces on FEC/FECR, the objectives of the present study were: (i) to further validate the pooling technique comparing FEC and FECR from individual sheep samples and pools of different size (5, 10 and 20 individual sheep samples), (ii) to assess the

effect of three different analytic sensitivities (10, 15 and 50) on individual and pooled samples using McMaster (analytic sensitivities = 15 and 50) and Mini-FLOTAC (analytic sensitivity = 10); and (iii) to determine the effect of the pooling on FECR.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Study design

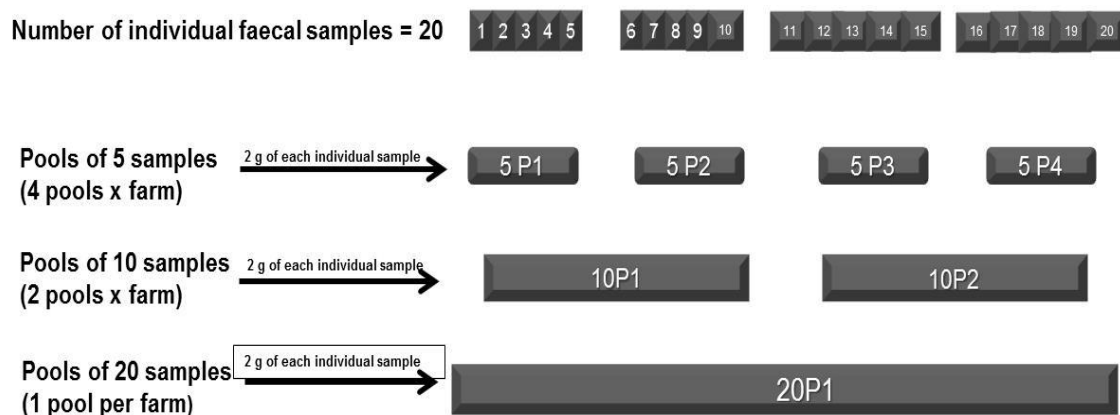
Between October and December 2012, a study was conducted on 10 sheep farms located in the Campania region of southern Italy. The animals (Fig. 2.2) on the farms were naturally infected with GIN (*Trichostrongylus* spp., *Haemonchus contortus* and *Teladorsagia circumcincta*) (Dipineto et al., 2013). On each farm, individual faecal samples (at least 20 grams) from 20 adult sheep (when possible) were collected, before (D0) and after (D14) anthelmintic treatment with albendazole 3.75 mg/kg (Valbazen 19 mg/ml - oral suspension, Pfizer). For each farm and at each time point (D0 and D14) the 20 samples were numbered from 1 to 20.



**Fig. 2.2.** Experimental animals.

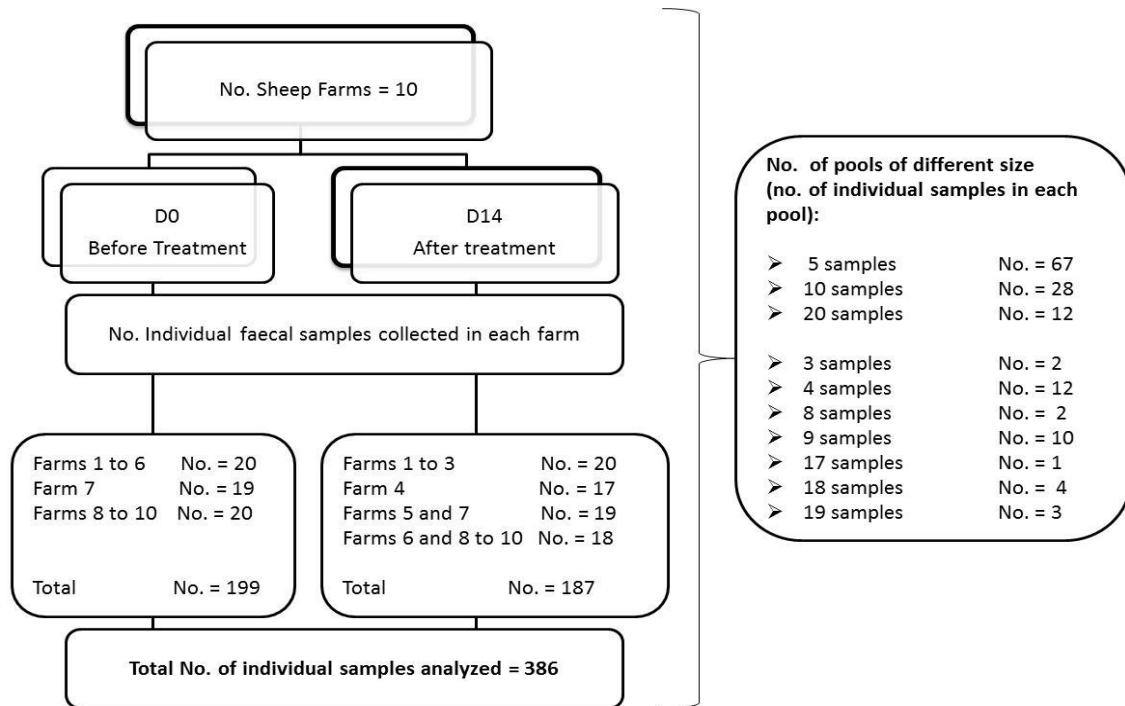
All faecal samples were individually processed by the McMaster and the Mini-FLOTAC techniques as described below.

In addition, for each farm and at each time point (D0 and D14), the faecal samples were pooled in pools of 5 individual samples ( $n = 4$ ), 10 individual samples ( $n = 2$ ) and 20 individual samples ( $n = 1$ ). All these pooled samples were prepared, using equal amounts from each individual faecal sample (2 grams) as shown in Figure 2.3.



**Fig. 2.3.** Procedure to obtain pools of 5, 10 and 20 individual sheep faecal samples.

The total number of sheep farms and the total number of individual and pooled samples across the assessment of the infection intensity and the efficacy trial (D0 and D14) are provided in Figure 2.4. It should be noted that the predefined pool sizes of 5, 10 and 20 could not be met when  $<20$  animals were sampled on a farm. Therefore, it was anticipated to have 80 pools of 5 (4 pools per farm x 10 farms x 2 occasions of sampling), 40 pools of 10 (2 pools per farm x 10 farms x 2 occasions of sampling) and 20 pools of 20 (2 pools per farm x 10 farms x 2 occasions of sampling) but the actual number of pools of different sizes is provided in Figure 5.4. However, it should be noted that in our analysis to verify differences in pool size, we considered all samples that met the predefined sample size. The pooled samples were stirred until homogenized. As for the individual samples, each pool was examined using McMaster and Mini-FLOTAC.



**Fig. 2.4.** Number of sheep farms, individual faecal samples and pools used for the study.

## 2.2.2 Parasitological examination

### *Modified McMaster technique*

The modified McMaster technique (MAFF, 1986) was performed using the following standard operating procedure (SOP). Three grams of faeces were put into a container and 42 ml of sodium chloride (NaCl, specific gravity = 1.200) were added (dilution ratio = 1:15). The faecal suspension was thoroughly homogenized and strained three times through a wire mesh (aperture of 250  $\mu$ m) to remove large debris. The strained suspension was collected in a bowl and thoroughly mixed by pouring it 10 times in one bowl to another. Then, 0.5 ml aliquots were added to each of the two chambers of a McMaster slide (<http://www.hawksley.co.uk/cell-count-glassware/05c-spec-chambers/>). After 10 minutes, the GIN egg counts were performed under the two grids (volume = 0.3 ml) and both chambers (volume = 1.0 ml) of the McMaster (Cringoli et al., 2004) under a light microscope using a 100x magnification. FEC values, expressed as EPG of GI strongyles, were obtained by multiplying the total number of eggs by 50 (McM50) or 15 (McM15).

### *Mini-FLOTAC technique*

The Mini-FLOTAC technique (Cringoli et al., 2013) was performed using the following SOP. Two grams of fresh faeces were put into the Fill-FLOTAC container and 38 ml of sodium chloride (NaCl, specific gravity = 1.200) were added (dilution ratio = 1:20). The suspension was then thoroughly homogenized using the homogenizer stick of the Fill-FLOTAC. The faecal suspension was then filtered through the Fill-FLOTAC, and used to fill the two chambers of the Mini-FLOTAC. After 10 minutes, the top part of the flotation chambers were translated and the Mini-FLOTAC was read under a light microscope using a 100x magnification. The analytic sensitivity of the Mini-FLOTAC basic technique was 10 EPG.

For both McMaster and Mini-FLOTAC, the quality of the parasitological examination was ensured by (i) analyzing the samples within an average of 7 hours of collection, (ii) verification of the density of the NaCl solution using a hydrometer, (iii) calibration of the scale weighing the faecal material, (iv) supervision of the pooling procedures and (iv) reading the McMaster and Mini-FLOTAC by two senior researchers.

### *2.2.3 Statistical analysis*

The statistical analyses were performed in the statistical software R (R Development Core Team, 2004). The level of significance was set at  $P < 0.05$  for all tests.

### *2.2.4 Comparison of individual and pooled samples for assessment of FEC and drug efficacy (FECD)*

The agreement in FECs between individual samples and pooled samples was verified by a permutation test (10,000 iterations) based on Pearson correlation coefficient and differences in EPG values for each of the pool sizes and FEC technique, separately.

The anthelmintic drug efficacy at each farm was measured by means of FECD using the formula below:

$$FECD (\%) = 100\% \times \left( 1 - \frac{\text{arithmetic mean of FEC at follow-up (D14)}}{\text{arithmetic mean of FEC at baseline (D0)}} \right)$$



As for FECs, the agreement in FECR between individual samples and pooled samples was verified by a permutation test (10,000 iterations) based on Pearson correlation coefficient and differences in FECR for each of the pool sizes and FEC technique, separately. The Tukey's method was applied for multiple comparisons.

#### *2.2.5 Comparison of diagnosis and assessment of drug efficacy across FEC techniques*

##### *Agreement in qualitative and quantitative diagnosis of GI strongyles*

The three copromicroscopic techniques (Mini-FLOTAC, McM15 and McM50) were compared qualitatively (sensitivity) and quantitatively (FECs). Sensitivity was calculated for each technique, using the combined results of the techniques as the diagnostic 'gold' standard. Therefore, the specificity of both Mini-FLOTAC and McMaster was set at 100%, as indicated by the morphology of the eggs. Differences in sensitivity between techniques were assessed by a permutation test taking into account the dependency of results within samples (10,000 iterations). The Tukey's method was applied for pair-wise comparison. The variation in sensitivity within each technique was explored by a logistic regression model, which was fitted for each of the techniques with their test result (positive/negative) as the outcome, and the mean FECs across techniques as covariate. The predictive power of this model was evaluated by the proportion of the observed outcome that was correctly predicted by the model. To this end, an individual probability >0.5 was set as a positive test result, and negative if different. Finally, the sensitivity for each of the observed values of FECs was estimated based on this model.

The agreement in FEC across the three techniques (Mini-FLOTAC, McM15 and McM50) was verified by a permutation test (10,000 iterations) based on Pearson correlation coefficient and differences in FECs. The Tukey's method was applied for multiple comparisons.

##### *2.2.6 Agreement in assessment of anthelmintic drug efficacy (FECR)*

We assessed the agreement across FEC techniques in classifying the drug efficacy into 'reduced' (= FECR <95% AND lower limit of the 95% confidence interval (LL of 95%CI) <90%), 'suspected to be reduced' (= FECR <95% OR LL of 95%CI <90%) and 'normal' (= FECR ≥95% AND LL of 95%CI ≥90%) as described by Coles et al.

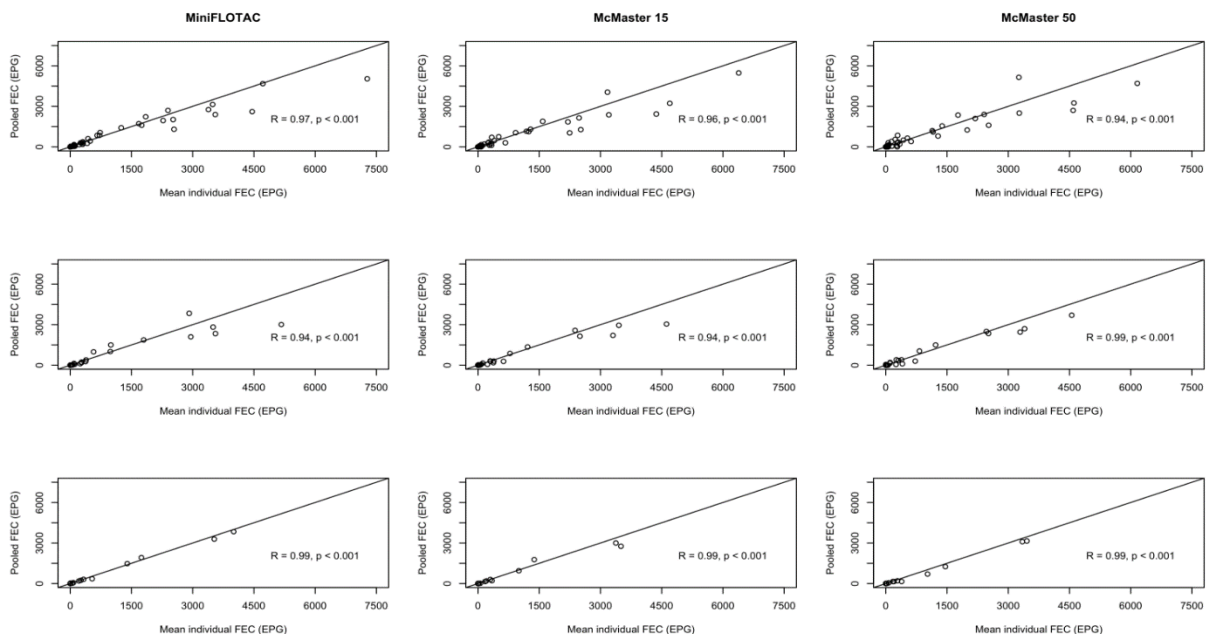
(1992). The 95%CI was based on a nonparametric bootstrap (10,000 iterations). The agreement in classifying the drug efficacy was evaluated by a permutation test (10,000 iterations) based on the Kappa Fleiss statistic.

## 2.3 RESULTS

### 2.3.1 Comparison of individual and pooled samples for assessment of FEC and FECR

#### Agreement in assessment of FECs

The correlation between FEC results of pooled samples and mean of individual FEC is illustrated by Figure 5.5. Overall, FEC results of pooled samples correlated positively with the mean FECs of individual samples, with high correlation coefficients (Rs), i.e.  $\geq 0.94$  ( $P < 0.0001$ ), regardless the pool size and the analytic sensitivity. The concordance plots illustrate a difference in level of agreement between the individual and pooled samples. This particularly for pool sizes of 10 and 20, for which FECs based on pooled samples result in lower estimates compared to FECs of individual samples as FECs increase (FECs based on pooled samples are located below the line of equality, slope 1).



**Fig. 2.5.** The agreement in FECs based on the examination of individual and pools of 5 (top row), 10 (middle row) and 20 (bottom row) samples for three different copromicroscopic techniques. R: Pearson's correlation coefficient. The straight line represents the line of equality (slope = 1).

The difference in FECs between pooled and individual samples is summarized in Table 2.1. Overall, examination of individual samples resulted in higher FECs with differences in FECs ranging from 20 to 99 EPG. No difference between methods was found. A significant difference in FECs was observed only for McM15 and when 10 samples were pooled. In this case, the mean difference between individual and pooled FEC was 99 ( $P = 0.05$ ).

**Table 2.1.** The difference in FECs between examination of pooled and individual samples for Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

Pair-wise comparison	Mean difference in FECs (EPG)		
	(P-value)		
	Mini-FLOTAC	McM15	McM50
Individual vs. pools of 5	90 (0.27)	91 (0.10)	56 (0.42)
Individual vs. pools of 10	86 (0.30)	99 (0.05)	68 (0.26)
Individual vs. pools of 20	20 (0.96)	50 (0.68)	76 (0.16)

*Agreement in assessment of FECR*

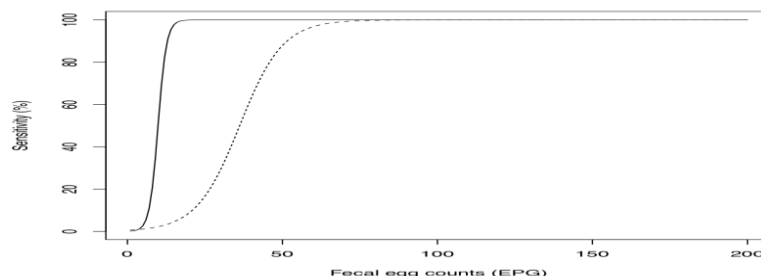
Table 2.2 summarizes per farm the FECR for the different pool sizes for each of the three copromicroscopic techniques. All methods performed well on all farms. With the exception of one farm (#4), pooling samples allowed for assessing FECR on all farms using all three FEC techniques. On this farm (#4), no FECR could be determined when using McM15 as the mean FECs of the pools post treatment were zero. This was also the case for pools of 10 and 20 when examined with McM50. With the exception of farms No. 2 and 3, FECR was 100% when calculated for

individual animals and across the different pool sizes (n = 5, 10 and 20 individual samples) and copromicroscopic technique (Mini-FLOTAC, McM15 and McM50). Given the low variation in FECR results, no attempts were taken to verify correlation, and differences in FECR between the three methods. However, noteworthy on Farm 3 FECR (%) was constantly below 100% using Mini-FLOTAC when calculated for individual animals and across the different pool sizes (n = 5, 10 and 20 individual samples). Mini-FLOTAC actually found resistance at the pool size of n=20, whereas the other methods missed it.

### 2.3.2 Comparison of diagnosis and assessment of drug efficacy across FEC methods

#### *Agreement in qualitative and quantitative diagnosis of GIN*

In 191 out of 386 (49.5%; 95% confidence intervals (95% CI) [44.4; 54.6]) samples GIN eggs were detected with at least one of the three copromicroscopic techniques. Mini-FLOTAC allowed for the detection of eggs in all the 191 samples (sensitivity = 100%, 95%CI [100; 100]). The sensitivities of McM15 and McM50 were 88.5% [84.0; 93.0] and 75.9% [69.9; 82.0], respectively. Mini-FLOTAC was more sensitive compared to both McM15 and McM50 ( $P < 0.001$ ). Furthermore, McM15 resulted in more sensitive test results compared to McM50 ( $P < 0.001$ ). Figure 5.6 indicates that both McM15 and McM50 often fail to detect low FECs, and that this was more pronounced for McM50. However, both McM15 and McM50 became equally sensitive compared to Mini-FLOTAC when FECs increased. For both methods, the model could correctly predict the observed test results in more than 95% of the cases.



**Fig. 2.6.** The predicted sensitivity derived from logistic regression for McMaster based on the examination of the entire slide (McM15; straight line) and of the grids (McM50; dashed line). For both methods, the model could correctly predict the observed test results in more than 95% of the cases.

**Table 2.2.** The agreement in FECR across different pool sizes (N) and copromicroscopic techniques (Mini-FLOTAC, McM15 and McM50).

Farm	Mini-FLOTAC				McM15				McM50			
	Ind	N = 5	N =10	N = 20	Ind	N = 5	N =10	N = 20	Ind	N = 5	N =10	N = 20
ID	FECR (%)				FECR (%)				FECR (%)			
1	100	100	100	100	100	100	100	100	100	100	100	100
2	99.6	100	100	100	99.1	100	100	100	98.6	100	100	100
3	98.8	97.3	99.1	91.4	98.5	98.9	90.0	100	100	100	83.3	100
4	100	100	100	100	100	100	100	-	100	100	-	-
5 to 10	100	100	100	100	100	100	100	100	100	100	100	100

Ind = individual samples

**Table 2.3.** Summarizes the agreement in FECs across the three copromicroscopic techniques. There was a significant positive correlation for each of the three pair-wise comparisons (Pearson's correlation coefficient >0.95,  $P < 0.001$ ). Mini-FLOTAC resulted in significant higher FECs compared to both McM15 and McM50, with a mean difference in egg counts of approximately 90 EPG ( $P < 0.001$ ). There was no significant difference in FECs across McMaster variants (mean difference of 3.9 EPG,  $P = 0.97$ ).

**Table 2.3.** The agreement in FECs across Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

Pair-wise comparison	Pearson correlation coefficient (P-value)	Mean difference in FECs (P-value)
Mini-FLOTAC vs McM15	0.98 (<0.001)	90.9 (<0.001)
Mini-FLOTAC vs McM50	0.97 (<0.001)	87.0 (<0.001)
McM15 vs McM50	0.99 (<0.001)	-3.9 (0.98)

*Agreement in assessment of anthelmintic drug efficacy (FEER)*

Table 2.4 summarizes per farm the number of animals included in the efficacy trial, mean FECs at baseline, FEER and the final interpretation on drug efficacy for each of the three copromicroscopic techniques. At least 17 animals per farm were sampled both before and after the administration of the drug. There was a wide variation in mean FECs at baseline, ranging from 52 to 4078 EPG for Mini-FLOTAC, from 21 to 3599 EPG for McM15, and from 29 to 3539 EPG for McM50. This was in contrast with the drug efficacy results, for which FEER were higher than 98% and drug efficacy was assigned as having 'normal' drug efficacy on all farms, and this was independent of the copromicroscopic techniques. Given this low variation in FEER results and the lack of disagreement in the final interpretation no attempts were taken to verify correlation, and differences in FEER and the final interpretation between the three techniques.

**Table 2.4.** The agreement in FECR across Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

Farm ID	No. samples	Mini-FLOTAC		McM15		McM50	
		Mean FEC at D0	FECR (95%CI)	Mean FEC at D0	FECR(95%CI)	Mean FEC at D0	FECR (95%CI)
1	20	1396	100(99.9; 100)	999	100(100; 100)	1023	100(100; 100)
2	20	261	99.6(98.7; 100)	173	99.1(96.7; 100)	175	98.6(94.4; 100)
3	20	536	98.8(97.5; 99.5)	341	98.5(96.4; 100)	388	100(100; 100)
4	17	52	100(100; 100)	21	100(100; 100)	29	100(100; 100)
5	19	1830	100(100; 100)	1444	100(100; 100)	1529	100(100; 100)
6	18	225	100(100; 100)	219	100(100; 100)	219	100(100; 100)
7	18	4078	100(100; 100)	3599	100(100; 100)	3539	100(100; 100)
8	18	3621	100(100; 100)	3428	100(100; 100)	3365	100(100; 100)
9	18	360	100(100; 100)	333	100(100; 100)	314	100(100; 100)
10	18	72	100(100; 100)	54	100(100; 100)	64	100(100; 100)

## 2.4 DISCUSSION

The present study provided new insights towards standardizing FEC/FECR on pooled faecal samples in sheep for the assessment of GIN infection intensity and anthelmintic drug efficacy. In particular, the effect of different pool sizes and analytic sensitivities on pooled FEC/FECR was evaluated.

Significant findings emerged regarding: (i) agreement between individual samples and pooled samples in assessment of FECs using the different analytic sensitivities (10 EPG using Mini-FLOTAC, 15 and 50 EPG using McMaster); (ii) agreement between individual samples and pooled samples in assessment of anthelmintic drug efficacy (FECR) using different analytic sensitivities; and (iii) qualitative (sensitivity) and quantitative (FECs) performance of the FEC methods.

First, regarding the agreement between individual samples and pooled samples in assessment of FECs, our findings showed that GIN EPG of pooled samples correlated positively with mean EPG of individual samples, with high correlation coefficients ( $\geq 0.94$ ) regardless pool sizes and analytic sensitivities. Despite this high correlation, there was an apparent, but insignificant underestimation of FECs when samples are pooled, which may need further attention. Nevertheless, in line with previous studies our findings support the potency of pooling strategy to reduce the workload in the laboratory. However, it is important to note that this study was not designed to verify to which extent the outcome of one pool of 5, 10 or 20 individual samples represents the average infection intensity at the flock level. Although, this would clearly further decrease the workload in both the field (fewer animals needed to be sampled) and the laboratory (only one FEC), this approach, as illustrated by Morgan et al. (2005), may resulted in a thwarted interpretation.

Second, concerning drug efficacy, with the exception of two farms, the present study showed FECR values of 100% when calculated for individual animals and across the 3 different pool sizes and analytic sensitivities. Therefore, as for FECs, the pooling approach worked very well also for FECR regardless of whether the pool was made up of 5, 10 or 20 individual samples, supporting previous studies. The very high drug efficacy found in the present study confirms that AR is rare in sheep of southern Italy, a region with a Mediterranean type of climate where the management system guarantees the maintenance of nematode populations *in refugia*, and anthelmintic use is limited



(Cringoli et al., 2008, 2009; Rinaldi et al., in press). However, the main limitation of these findings on FECR is represented by the high efficacy (100% in most of farms) of anthelmintics found in the present study. Therefore, further studies are required to assess the validity of FECR on pooled faecal samples also in settings where the efficacy of anthelmintics is less than 95% and AR is suspected.

Third, regarding the sensitivity of the FEC techniques, as expected, our findings showed that Mini-FLOTAC was more sensitive compared to the two variants of McMaster (McM15 and McM50) for the diagnosis of GIN in sheep (100% vs 88.5% vs 75.9%). Both McM15 and McM50 often failed to detect low GIN EPG but became equally sensitive compared to Mini-FLOTAC when FECs increased, thus confirming the findings of other studies on comparison of FEC techniques (e.g. Rinaldi et al., 2011; Levecke et al., 2011, 2012a,b). Mini-FLOTAC also resulted in significant higher FECs compared to both McMaster variants, with a mean difference in egg counts of approximately 90 EPG ( $P < 0.001$ ). However, it remains unclear to which extent this difference has a biological and/or practical impact. There is still no information available on the EPG threshold above which it is advisable to intervene with a specific control program, for example using a targeted treatment or a targeted selective treatment approach. All these questions and considerations underline that it is imperative to pay more attention to the final interpretation of FECs prior to recommend any FEC technique and any analytic sensitivity.

In addition, there is a lack of information regarding the actual cost-effectiveness of the pooled approach in copromicroscopy. It would be therefore advisable to conduct a comparative cost assessment study of individual and pooled FEC/FECR taking also in consideration the effect of different pool sizes and analytic sensitivities (e.g. McMaster *versus* Mini-FLOTAC). Valid examples of reliable and precise methodologies for assessing cost-effectiveness in copromicroscopy can be taken from the literature (e.g. Levecke et al., 2009; Speich et al., 2010).

Overall, the results of our study showed that pooling faecal samples can be used for FECs and FECR. Our findings are in line with recent studies on the same topic. As an example, pooled FEC was successfully used in horses (Eysker et al., 2008) for monitoring helminth control. Furthermore, Daniel et al. (2012) used FECR on pooled samples to assess the efficacy of triclabendazole against *Fasciola hepatica* in sheep farms in the UK. Concerning public health, Mekonnen et al. (2013) highlighted that

pooling stool samples is a promising approach for rapidly assessing infection intensity of soil transmitted helminths in humans as well as for drug efficacy studies. Finally, in their recent computer-based simulation study, Calvete and Uriarte (2013) suggest that the diagnostic performance of the FECR test (also using a pooled approach) should be re-evaluated and the recommendations of the W.A.A.V.P. should be updated as already reported in Levecke et al. (2012a).

In conclusion, the present study highlighted that pooling ovine faecal samples is a rapid procedure that holds promise as a cost-effective, but at the same time accurate strategy for assessing the intensity of GIN (FEC) in sheep as well as anthelmintic efficacy (FECR). However, further research is required (i) to determine biological and epidemiological significance of FECs in sheep farms in order to establish the EPG thresholds for control programs; (ii) to verify in-depth the cost-effectiveness of pooled FECs compared to individual FECs; (iii) to optimize and standardize the methodology of pooling faecal samples; (iv) to verify the validity of the pooled FECR test also in settings where anthelmintic efficacy is less than 95%; and (v) to assess the performance of pooling FEC for copromicroscopic diagnosis of helminths other than GIN and protozoa in sheep as well as in other livestock species.

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## CHAPTER 3

### *A comparison of the FECPAK and Mini-FLOTAC faecal egg counting techniques\**

\*Based on the manuscript: Godber, O.F., Phythian, C.J., Bosco, A., **Ianniello, D.**, Coles, G., Rinaldi, L., Cringoli, G. 2015. A comparison of the FECPAK and Mini-FLOTAC faecal egg counting techniques. *Vet Parasitol.* 30; 342-345.



### **3.1 INTRODUCTION**

The key feature of both the FECPAK and the Mini-FLOTAC techniques is the removal of the centrifugation step, which is designed to increase the speed and simplicity of egg counts and allowing for on-farm application. To the authors' knowledge, the accuracy and precision of the FECKPAK (where each egg counted represents 30 epg) and Mini-FLOTAC (where each egg counted represents 5 epg) egg counting techniques have not been previously compared. This study aimed to compare whether FEC results determined by FECPAK and Mini-FLOTAC significantly differed. This paper presents the findings of a laboratory trial which compared the degree of measurement error and precision of these two FEC methods using sheep faeces contaminated with known numbers of nematode eggs.

### **3.2 MATERIALS AND METHODS**

Faecal samples with positive and negative FEC were collected directly from the rectum of adult ewes in the Campania region of southern Italy. Absence of nematode eggs in the negative samples was confirmed by their thorough homogenisation and subsequent analysis by the FLOTAC basic technique where each egg counted represents 1 epg (Cringoli et al., 2010). Nematode eggs were extracted from the positive samples using mass extraction and diluted. Following confirmation of egg concentration this egg suspension was added to the negative faeces and thoroughly homogenised to minimise error in the distribution of eggs within the sample to achieve FECs of 10, 50, 200, 500 and 1000 epg. For each level of egg density three subsamples were prepared. Four FECPAK or Mini-FLOTAC chambers were prepared and read for each subsample by four independent researchers to account for potential error in egg distribution within the

samples and operator counting error. In total 12 FECs were performed at each level of egg density using each technique. Each egg counted represented 60 epg, 22 epg and 10 epg for the individual FECPAK grid, FECPAK chamber and Mini-FLOTAC grids respectively and 30 epg, 11 epg and 5 epg for the entire slide or disk (two grids or chambers; see Table 3.1.).

**Table 3.1** The dilution ratio, volume of suspension and analytic sensitivity in eggs per gram (epg) of the FECPAK and Mini-FLOTAC faecal egg counting techniques.

<b>Technique</b>	<b>Area counted</b>	<b>Dilution ratio</b>	<b>Volume of suspension (ml)</b>	<b>Analytic sensitivity (epg)</b>
<b>FECPAK</b>	1 x grid	1:30	0.5	60
	2 x grid	1:30	1.0	30
	1 x chamber	1:30	1.4	22
	2 x chamber	1:30	2.8	11
<b>Mini-FLOTAC</b>	1 x chamber	1:10	1.0	10
	2 x chamber	1:10	2.0	5

### 3.3 DATA ANALYSIS

Statistical analysis was performed in R version 2.15.2 under the R studio interface version 0.98.501. For each FEC technique at each level of egg density the percentage error  $((\text{expected FEC} - \text{observed FEC}) / \text{expected FEC} * 100)$  was calculated to assess

the level of over- or under- estimation of FEC result (measurement error). Variability (precision) was assessed from the interquartile range. The Wilcoxon signed rank test (V) assessed pair-wise differences between the different faecal egg counting techniques with a Bonferonni post-hoc correction for multiple testing. Spearman rank correlation ( $\rho$ ) examined for any association between egg density and the direction of measurement error of each faecal egg counting technique. Significance testing was set at  $p < 0.05$ .

### **3.4 RESULTS**

#### *3.4.1. Comparison of the Mini-FLOTAC and FECPAK*

A total of 120 FECs were performed; 12 for each technique (Mini-FLOTAC and FECPAK) at each level of egg concentration. At all egg densities the Mini-FLOTAC shows fewer counting errors than the FECPAK which tended to underestimate FECs (Table 2). The FECPAK significantly underestimated egg counts;  $V = 565$ ,  $n = 59$ ,  $p < 0.01$ , with the largest measurement error occurring between 50 ( $V = 9$ ,  $n = 11$ ,  $p = 0.019$ ) and 200 epg ( $V = 1$ ,  $n = 11$ ,  $p = 0.003$ ). In contrast, Mini-FLOTAC observed FEC did not differ significantly from expected at any level of egg density ( $p > 0.05$ ; Table 2.2).

**Table 3.2** A comparison of the measurement error, accuracy and precision of the Mini-FLOTAC and FECPAK egg counting techniques at different levels of egg density

Expected egg density (epg)	Technique	Median observed count (epg)	Measurement error (median percentage error in FEC, %)	Precision (interquartile range of percentage error)
10	Mini-FLOTAC	10	0.00	100.0
	FECPAK grid	0	-100.0	300.0
	FECPAK chamber	0	-100.0	100.0
50	Mini-FLOTAC	50	-3.85 <sup>a</sup>	31.3
	FECPAK grid	30 <sup>*</sup>	-42.3 <sup>b</sup>	57.7
	FECPAK chamber	11 <sup>**</sup>	-78.9 <sup>b</sup>	26.4
200	Mini-FLOTAC	198	-1.25 <sup>a</sup>	13.8
	FECPAK grid	90 <sup>**</sup>	-55.0 <sup>b</sup>	45.0
	FECPAK chamber	88 <sup>**</sup>	-56.0 <sup>c</sup>	22.0
500	Mini-FLOTAC	513	2.50 <sup>a</sup>	8.00
	FECPAK grid	526	-1.25 <sup>a</sup>	40.5
	FECPAK chamber	407 <sup>**</sup>	-56.0 <sup>b</sup>	27.0
1000	Mini-FLOTAC	1008	0.75 <sup>a</sup>	11.0
	FECPAK grid	1050	5.00 <sup>a</sup>	47.2
	FECPAK chamber	748 <sup>*</sup>	-25.2 <sup>b</sup>	24.5

12 samples were analysed for each technique at each level of egg density; percentage error calculated by (expected FEC – observed FEC) / expected FEC \* 100; epg = eggs per gram; \* = significant difference ( $p < 0.05$ ) between observed and expected FEC; \*\* = highly significant difference ( $p < 0.01$ ) between observed and expected FEC; if superscript letters of results differ then medians of techniques differ significantly ( $p < 0.05$ ).

Precision is inversely correlated with the interquartile range of percentage error. Table 2 shows that Mini-FLOTAC had the greater precision at all levels of egg density. For both the Mini-FLOTAC and FECPAK, precision was lowest at an egg density of 10 epg, which improved greatly at 50 epg but shows little further improvement with increasing egg density beyond this point.

#### *3.4.2 Different methods of counting the FECPAK slide*

Eggs were counted in both the gridded area and the total chamber of the FECPAK slides. For both FECPAK techniques, measurement error for FECKPAK grid ( $\rho = 0.44$ , d.f. = 58,  $p < 0.001$ ) and chamber ( $\rho = 0.53$ , d.f. = 58,  $p < 0.001$ ) improved significantly with egg density. FECPAK chamber FECs produced greater errors than the grid FECs at all levels of egg density with observed chamber FECs significantly underestimated the expected FEC at all levels of egg density above 10 epg ( $p < 0.05$ ). In comparison, the observed FECPAK grid FECs only significantly underestimated the expected FEC at 50 and 200 epg. Precision was greater for the FECPAK chamber FECs than the FECPAK grid FECs at all levels of egg density (Table 3.2).

### **3.5 DISCUSSION**

This study examined the diagnostic performance of the Mini-FLOTAC and FECPAK faecal egg counting techniques in terms of measurement error (the amount of over- or under- estimation of FEC result), and precision (variability in results) at a range of egg densities (10 to 1000 epg).

At lower egg densities, both methods had a tendency to underestimate FECs. However, results indicate that precision of the Mini-FLOTAC was greater than that of the FECPAK at all levels of egg density. This may reflect the differing sensitivities since egg counted

represents 5 epg with the Mini-FLOTAC compared to 30 epg with FECPAK. Torgerson et al. (2012) previously identified that applying a larger multiplication factor to raw FECs, as is necessary with the FECPAK, leads to a greater inflation of variation (reduced precision) in the final FEC. Although underestimated FECs were not observed at the 10 epg level in this study, they would be expected at the lower egg concentrations of 10 and 50 epg with the FECPAK as each egg counted represents 30 epg.

Significant under-estimation of FEC occurred when the entire FECPAK chamber was examined rather than limited to the gridded area. This is in agreement with Cringoli et al. (2004) who observed aggregation of eggs to the centre of McMaster slides and Morgan et al. (2005) who described the Poisson distribution of nematode eggs in faecal suspensions. Only counting eggs in the gridded area appears to account for this aggregation at higher levels of egg densities; the number of eggs present at lower densities, however, was still underestimated. Therefore, although counting of the entire chamber rather than solely the gridded area is considered to improve precision due to the reduced dilution factor (Torgerson et al., 2012), the greater degree of measurement error seen in the present study clearly outweighs this benefit and supports the approach recommended by FECPAK to count the number of eggs under one or both gridded areas rather than the full chamber (Coles, 2003).

### **3.6 CONCLUSION**

This study compared FECPAK and Mini-FLOTAC apparatus on sheep faeces. In contrast to the FECPAK, the Mini-FLOTAC FEC results did not differ significantly to the expected result at any egg concentration. This study shows that the Mini-FLOTAC can be considered as an alternative tool offering reduced measurement error and a higher level of precision and may be particularly relevant to studies assessing anthelmintic efficacy.

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## CHAPTER 4

### *The recovery of added nematode eggs from sheep faecal by three methods*

\*Based on the manuscript: **Ianniello, D.**, Bosco, A., Rinaldi, L., Coles, G., Cringoli, G. The recovery of added nematode eggs from sheep faecal by three methods. *Vet. Parasitol.*, 2015, in press.

## 5.1 INTRODUCTION

To improve the accuracy of nematode egg counting in sheep faecal samples the accuracy and precision of three faecal egg counting (FEC) methods were compared, Min-FLOTAC combined with Fill-FLOTAC, McMaster and Cornell-Wisconsin. Known numbers of eggs extracted from ovine faeces were added to egg free ovine faeces to give counts of 10, 50, 200 and 500 epg. In addition, the sampling accuracy of the new method of Fill-FLOTAC and its use in two different sampling procedures were evaluated.

## 5.2 MATERIALS AND METHODS

### 4.2.1 Faecal sampling

Faecal samples with positive and negative FEC were collected directly from the rectum of adult sheep bred in the Campania region of southern Italy. Each sample was counted 5 times by the FLOTAC Basic technique (Cringoli et al., 2010) with an analytic sensitivity of 1 EPG (eggs per gram) to determine the presence/absence of nematode eggs. Nematode eggs were isolated from the positive ovine samples using flotation in saturated sodium chloride (modified from Coles et al., 2006) and diluted. Ten aliquots of 0.1 ml each were taken and the number of eggs counted (Godber et al., 2015). The eggs suspensions were added to the negative faeces and thoroughly homogenized to give FECs of 10, 50, 200 and 500 eggs per grams. The preparation of the samples was performed by the same individual for each repetition to minimize error.

### 4.2.2 FECs methods

Each sample was analyzed by three FEC techniques: Mini-FLOTAC combined with Fill-FLOTAC (Cringoli et al., 2014; as described in Rinaldi et al., 2014); Cornell Wisconsin technique (Egwan and Slocombe, 1982;) modified McMaster technique (MAFF, 1986; as described in Rinaldi et al., 2014) with eggs being counted in both the gridded area and the total chamber of

the McMaster slides. The weight of faeces used, dilution ratio, reading volume and, analytic sensitivity are shown in Table 4.1. Twelve replicates were used with each method.

**Tab.4.1.** The weight of faeces, dilution ratio, reading volume, reading area and analytic sensitivity) of Mini-FLOTAC, two versions of McMaster and Cornell-Wisconsin egg counting chambers.

<b>FEC Techniques</b>	<b>Amount of feces used (grams)</b>	<b>Dilution ratio</b>	<b>Reading Volume (ml)</b>	<b>Reading Area (mm<sup>2</sup>)</b>	<b>Analytic sensitivity (EPG)</b>
<b>Mini-FLOTAC</b>	5	1:10	1.0	648	5
<b>Mc MASTER <i>grid</i></b>	3	1:15	0.30	200	50
<b>Mc MASTER <i>chamber</i></b>	3	1:15	1.0	648	15
<b>Conrell-Wisconsin</b>	5	1:3	15	324	1

### 4.3. FILL-FLOTAC SYSTEM

Fill-FLOTAC is a simple accurate method that eliminates the need to weigh samples and also filters larger debris out of the suspension. This kit facilitates the first four consecutive steps of the Mini-FLOTAC technique, i.e. collection (including weighing), homogenization, filtration and filling (Barda et al. 2013). The repeatability of the 5 g size of Fill-FLOTAC to measure 5 grams of faeces using sheep samples was measured 10 times .

## 4.4 Results

### 4.4.1 Comparison of FECs methods

The study involving 192 counts showed that at all egg densities the Mini-FLOTAC had fewer counting errors than the other techniques which tended to underestimate FECs (Table 4.2. a, b).

**Table 4.2a.** Mean percent of GIN eggs (% , mean (x)) recovered by Mini-FLOTAC, McMASTER and Cornell-Wisconsin from sheep faeces containing a predetermined number of nematode eggs extracted from sheep faeces.

EPG	Mini-FLOTAC		Mc MASTER grid		Mc MASTER chamber		Wisconsin	
	%±SD	$\bar{X}$ +SD	(%±SD)	$\bar{X}$ +SD	(%±SD)	$\bar{X}$ +SD	(%±SD)	$\bar{X}$ +SD
<b>10</b>	108±29	10.8±3	42±144	4.2±14	63±77	6.3±8	25±10.9	2.5±1
<b>50</b>	96±8	48±4	83±94	42±47	73±53	36±27	23±6.9	12 ±3
<b>200</b>	99.8±12	199±24	87.5±27.2	175±54.4	81.9±24.0	164±48	60.8±5.9	122±12
<b>500</b>	99.6±5	498±27	104.2±23.5	520.8±117.7	91±12	454±62	49±3	245±15

**Table 4.2b.** Prevalence of the Mini-FLOTAC, McMASTER and Cornell-Wisconsin from sheep faeces containing a predetermined number of nematode eggs extracted from sheep faeces.

EPG	Mini-FLOTAC %	Mc MASTER grid %	Mc MASTER chamber %	Wisconsin %
<b>10</b>	100	10	50	100
<b>50</b>	100	50	80	100
<b>200</b>	100	100	100	100
<b>500</b>	100	100	100	100

#### 4.4.2 *Different methods of counting the McMaster slide*

McMaster chamber FECs produced greater errors than the grid FECs at all egg contaminations. Precision (% eggs extracted) was greater for the McMaster grid FECs than the McMaster chamber FECs at all levels of egg density (except for 10 EPG concentration) (Table 2 a), but the prevalence (% positive) was greater for McMaster chamber than the McMaster grid FECS at all levels of egg density.

#### 4.4.3 *Fill-FLOTAC sampling*

The capacity to measure 5 grams of faeces by Fill-FLOTAC 5g collector, using sheep samples, was very precise. Indeed weighing sheep faeces had an average of 5.1 grams ( maximum value revealed 5.1, minimum value revealed 4.8).

### **4.5. Discussion**

The Mini-FLOTAC and Cornell-Wisconsin techniques showed recovery rates (% positive) of 100% at each level of contamination, while McMaster showed positivities only for contamination above 200 EPG. The accuracy (% eggs extracted) of the Mini-FLOTAC was always greater than 96%, reaching 100% at levels of contamination than 50EPG. At lower egg densities, McMaster and Cornell-Wisconsin methods had a tendency to underestimate FECs. However, results indicate that precision of the Mini-FLOTAC was greater than that of the McMaster and Cornell-Wisconsin at all levels of egg density. This may reflect the differing sensitivities since egg counted. The very poor performance of the Cornell-Wisconsin method indicates that this should not be used in the future for counting nematode eggs. For many purposes the McMaster technique will be adequate if egg counts are greater than 200 epg, but it is not satisfactory for lower counts which could be important if looking for the beginning of

anthelmintic resistance. Unless centrifugation is added to remove fine debris, which darkens the samples, eggs can be more difficult to see than in Mini-FLOTAC where translations takes the eggs away from the main volume of the well. Since Mini-FLOTAC is also the most sensitive technique it is obviously the test of choice. Suggestions that it takes longer to count than the McMaster technique when egg counts are high is easily solved by only counting one well or even half of one well and using a different multiplication factor.

#### **4.6. Conclusion**

The Mini-FLOTAC method is the best and easiest eggs counting method for sheep and nematode eggs from other species and it should therefore be considered to become the world standard. Combined with Fill-FLOTAC which provides an accurate method of weighting without need for a balance and filtering out debris, the two procedures together make the best method for use for sample preparation on the farm as well as in the laboratory.

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# CHAPTER 5

## Overall Discussion

### Gastrointestinal nematode faecal egg counts in small ruminants: present assessments and future perspectives

#### 5.1. INTRODUCTION

Infections with gastrointestinal nematodes (GIN) are a major cause of economic losses in ruminant livestock production worldwide, primarily through subclinical disease (Charlier et al., 2014). Despite this, diagnosis of GIN is still neglected by the scientific community (Rinaldi and Cringoli, 2014).

The present thesis provided important insights into the coprological diagnosis of GIN infections in small ruminants with particular emphasis on the significance, interpretation and limitations of different faecal egg count (FEC) techniques and the faecal egg count reduction test (FECRT).

Significant findings emerged regarding: i) the value of pooled faecal samples to assess GIN infection intensity (FEC) and anthelmintic efficacy (FECR) in sheep; ii) the calibration and performance of different FEC techniques.

An in-depth analysis of FEC/FECR, considering also limitations and gaps reviewed in Chapter 1, has important implications towards the achievement of a proper diagnosis of GIN in small ruminants, particularly when FEC/FECR are used in epidemiological surveys, anthelmintic drug efficacy studies and monitoring of control programs.

Overall, the results of the studies presented in Chapters 2 to 4 highlighted that: i) pooling faecal samples using the recently developed Mini-FLOTAC technique is an accurate and rapid procedure that holds promise a valid strategy for assessing FECs and FECR of GIN in sheep (Chapter 2, Rinaldi et al., 2014a); ii) Mini- FLOTAC, combined with Fill-FLOTAC are the most accurate FEC technique and sampling method, respectively, for reliable GIN egg counts in sheep (Chapter 3 and 4, Godber et al., 2015).

In the next paragraphs we will discuss the importance of a “continuing and up-to-date education” on FEC/FECR to parasitologists, veterinarians and farmers in Italy, Europe and beyond. Recognizing this challenge, standardization of existing procedures, and innovating, validating and applying new tools and strategies, will hopefully foster and sustain long-term control of GIN infections in small ruminants.

Explicitly, the following issues will be discussed: (i) the role of FEC/FECR for the detection of anthelmintic resistance; (ii) the role of FEC/FECR to perform targeted (selective) treatments; (iii) the need for other diagnostic tools in combination with FECs; (iv) how to promote FEC/FECR among practitioners and farmers; (v) the strategy of FEC/FECRT in the Campania region (southern Italy) and finally (vi) the future of GIN egg counts in small ruminants.

## **5.2. THE ROLE OF FEC/FECR FOR THE DETECTION OF ANTHELMINTIC RESISTANCE**

### *5.2.1. Background*

Anthelmintic resistance (AR) is now widespread in all the major GIN species infecting sheep and goats. Since the development of new, broad-spectrum anthelmintics may be not for the near future, there is a major need to preserve those that we currently have at our disposal. Hence, monitoring the drug-susceptibility and -resistance status of GIN populations in small ruminants must be a high priority and should be an important component of integrated management strategies (Charlier et al., 2014). Early detection of AR is crucial to avoid exponential increase of AR and associated production losses. The current de-facto test for AR is the FECRT. It is the only method that allows assessing drug efficacy of all anthelmintics, against all GIN species, and without sacrificing the animals (McKenna, 2013). In addition, it is simple and relatively easy to perform (Demeler et al., 2012) compared to the other *in vivo* and *in vitro* methods currently available for detecting AR.

### *5.2.2. Drawbacks*

A first limitation of the FECRT is the time and cost to conduct FECs on a representative number of individual animals in a representative number of farms at local and regional levels. As demonstrated in this thesis, the use of pooled samples to detect AR (Chapter 4, Rinaldi et al., 2014a) is a valid alternative to reduce workload/cost for the diagnosis and to encourage uptake of the FECRT by veterinarians and farmers. With a more user-friendly FECRT method and a pooling approach, sampling of larger number of farms can be performed thus providing a more accurate picture of AR in sheep at a larger scale.

Secondly, an issue to consider when conducting surveys on AR in pilot regions is the definition of an optimal strategy for farm sampling taking into consideration the costs and the stratification of farms according to environmental conditions and management practices. This requires accurate and efficiently collected information on predisposing factors for AR, related, for example, to the landscape, the levels of infection, the management system, the treatment regimes, etc.

Thirdly, since interpretation of FECRT is of paramount importance, user-friendly computer-based systems for easy calculations of “efficacy”, or “resistance” are needed. To this aim, Torgeson et al. (2014) developed a new R package "eggCounts" with a user friendly web interface that incorporates both sampling error and over-dispersion between animals to calculate the true egg counts in faecal samples, the probability distribution of the true counts and summary statistics such as the 95% uncertainty intervals. Based on a hierarchical Bayesian framework, the software also rigorously estimates the percentage of FECR and the 95% uncertainty intervals of data generated by a FECRT.

Fourthly, confounding factors unrelated to the presence of AR, such as inappropriate drug quality, drug administration (e.g. under-dosing) or host-related factors (e.g. diarrhoea) may complicate the interpretation of FECRT results (El-Abdellati et al., 2010) and should be carefully analyzed taking into account the concept of the “Good Practices of Treatment” (Taylor, 2012) aimed at using anthelmintics properly and effectively.

### *5.2.3. Recommendations*

Better recommendation should be given to the veterinarians/farmers based upon the results of the FECRT in order to provide a rationale guidance depending on whether AR is absent (not present), emerging or present in a farm.

Where AR **is not present**, as for example in southern Italy (Rinaldi et al., 2014b), farmers could be advised to continuing the control strategies and management practices currently used in their farms (use of targeted treatments based on two anthelmintic treatments per year, rotation of different drugs, correct drenching, low movement of sheep between farms, etc.). However, they should be also advised to routinely (every 6 months in the periods March-April and September-October, i.e. at turn out and turn in) monitor anthelmintic efficacy by FEC/FECR using a very accurate diagnostic technique in order to detect (early) changes in susceptibility in their sheep worm populations.

Instead, where **AR is emerging** farmers should be advised to reduce the number of anthelmintic treatments per year, to reduce the number of animals to be treated, to avoid clean grazing strategies, to perform correct quarantine strategies for in-coming animals and to monitor the progress of AR by performing regular (every 4-6 months in the periods March-April and September-October, i.e. at turn out and turn in) FEC/FECR on their farm (Coles, 2002).

Finally, **where AR is present**, farmers should be advised to change the anthelmintic class, and to perform FEC/FECR to assess efficacy of the new anthelmintic class (or combination) used and a regular (every 6 months) follow-up.

### **5.3. THE ROLE OF FEC/FECR IN THE ERA OF TARGETED (SELECTIVE) TREATMENTS**

#### *5.3.1. Background*

Infections by GIN are arguably the most important causes of suboptimal productivity in sheep. Hence their sustainable control is a prerequisite for economically efficient farming (Morgan et al., 2013). Two important concepts were recently introduced to promote the sustainable use of anthelmintics (Kenyon and Jackson, 2012): (i) targeted treatments (TT) where the whole flock is treated based on knowledge of the risk, or parameters that quantify the severity of infection, and (ii) targeted selective treatments (TST), where only individual animals within the grazing group are treated, based on a single, or a combination of, treatment indicators such as FECs, weight gain, body condition score, and milk yield (reviewed in Charlier et al., 2014). Although often criticized as treatment indicator, FECs provide a relatively direct estimate of parasite abundance and can be used for TT and TST if interpreted in a rational and appropriate way. It should be noted that pooled FECs can be used for monitoring the efficacy of TT (Cringoli et al., 2008), whereas individual FECs are mandatory for TST (Cringoli et al., 2009; Kenyon et al., 2009) and this may therefore increase the workload and costs for sampling and laboratory procedures.

#### *5.3.2. Drawbacks*

For both TT and TST, rigid interpretation of FEC results can be potentially misleading (Sargison, 2013) because there are no widely consented FEC thresholds for treatment decisions. Some authors suggest that less than 500 EPG is considered a low level of GIN infection, between 500 and 1500 EPG as moderate to high, and more than 1500 EPG as a high

level of infection (Hansen and Perry, 1994). According to other authors FECs of  $\geq 200$  EPG is regarded to indicate a significant worm burden and is used as a basis for the decision for anthelmintic treatment ([www.wormboss.com.au](http://www.wormboss.com.au)). Another problem related to the FEC thresholds is due to the fact that EPG values will change according to the fecundity of the GIN species infecting the animals. Indeed, as an example, in areas where co-infection with many GIN species occurs, the presence of high fecund species (e.g. *Haemonchus contortus*) will produce high EPGs, whereas the presence of less fecund species (e.g. *Teladorsagia circumcincta*) will result in low EPGs (Roeber et al., 2013).

### 5.3.3. Recommendations

To gain maximal information from FECs, strict thresholds for treatment should not be applied. In addition, FECs alone should not be used to guide treatment decisions, but be always interpreted in conjunction with information about the epidemiology of GIN in the region as well as the nutritional status, age, level and objective of production, and management of sheep/goats in a flock (McKenna, 2002).

In order to obtain useful information from FECs for treatment decisions, baseline FEC data (i.e. longitudinal data) should be monitored at farm and regional levels. Indeed, the timing of treatments based on monthly FEC trends seems to be crucial for the strategic and production efficacy of control strategies (Cringoli et al., 2008, 2009). At this regard, a series of studies have been performed within the PARASOL (EU-FP6) and GLOWORM (EU-FP7) projects, in order to evaluate the benefits of TT and TST approaches in the ovine sector in the Campania region of southern Italy. The TT scheme is based on two treatments timed in relation to parturition, i.e. the first in the periparturient period and the second at the mid/end of lactation. These periods for treatments have been chosen based on longitudinal data on FECs collected for several years on different pilot farms in the region. Data analysis showed that high values of GIN EPGs are observed during the periparturient period and mid/end of lactation (Cringoli et al., 2008). The benefit in milk yield, weight of lambs and reduced GIN egg output of the treated animals provide clear evidence that TT could improve animal performance and reduce pasture contamination. For these reasons, this TT scheme is now fairly integrated into routine dairy sheep farm management in southern Italy (reviewed in Charlier et al., 2014).

Similarly, studies in UK Morgan et al. (2012) and Australia (Besier and Love, 2003) showed that the timing of treatments can be a significant factor in AR development if treatments are

performed when seasonal climatic factors or management routines favour the survival of resistant GIN species (Besier and Love, 2012).

## **5.4. NEED FOR OTHER DIAGNOSTIC TOOLS IN COMBINATION WITH FEC**

### *5.4.1. Background*

In addition to commonly used FEC techniques, a number of biochemical, immunological and pathophysiological approaches have been developed for GIN and can be used in combination with FECs. These methods are mainly based on the detection and measurement of morbidity parameters that might be indicative of GIN infections (reviewed in Demeler et al., 2012 and Roeber et al., 2013).

### *5.4.2. Drawbacks*

Among the **biochemical parameters** (pepsinogen and gastrin), studies performed in cattle showed an increase in serum pepsinogen related to mucosal damage by developing larval stages of *Ostertagia* and a stimulation of G-cells by GIN has been related to an increase of gastrin concentration in infected animals. However, Berghen et al. (1993) reviewed the value and application of pepsinogen, gastrin and antibody responses as diagnostic indicators for ostertagiosis in cattle and identified a number of potentially limiting factors as the low specificity of this approaches.

**Direct immunological methods** (e.g. coproantigen-ELISAs) provide direct evidence of an infection and can be based on the detection of parasite antigens present in the circulation and/or excreta from infected hosts. However, the main limitation of these methods are based on the fact that shared antigenic composition of closely related GIN species often leads to cross-reactivity (Eysker and Ploeger, 2000). Indeed, the diagnostic performance of copro-ELISAs are often promising under experimental conditions, but cross-reactivity, faecal components interfering with the reactivity and the loss of antigens in faeces have been reported under field conditions (Johnson et al., 1996).

**Indirect immunological methods** are usually based on the detection of anti-GIN antibodies or cell-mediated immune responses in infected hosts. Various serum ELISAs are reported in Demeler et al. (2012) for the detection of infections by *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* or *Oesophagostomum*. However, GIN possess a huge variety of antigens, and there is limited information on which stages and antigens are actually responsible for eliciting immune responses (Berghen et al., 1993). Antibody detection from

serum has several disadvantages, including that it cannot distinguish between a current and past infection. It should be noted that a commercially available saliva test for the detection of nematode infection in sheep has been recently reported. The test measures antibodies (IgA), which are considered to be directed against parasite larvae in the gut mucous of sheep (Demeler et al., 2012)

FECs can also be used in combination with **advanced molecular technologies** (PCR, RT-PCR, MT-PCR) (reviewed in Roeber et al., 2013). However, although the use of molecular-based technologies offer the potential for multiplex, high-throughput diagnosis of GIN, these tools are not used in the routine practice yet.

**Finally, morbidity parameters** as anemia, diarrhea, body scoring and weight gain have also been employed in combination with FECs. For example, the FAMACHA system (van Wyk and Bath, 2002) can be used to identify sheep and goats suffering from anemia (likely caused by *Haemonchus*), and a diarrhea index (DISCO) can be a good indicator of actual nematode infection during the summer and autumn in a temperate climate (Cabaret, 2004). In addition, body condition scoring (BODCON) (van Wyk and Bath, 2002), and weight gain (LIVGAIN) are also potential methods for identifying animals requiring anthelmintic treatments. However, the value of these methods varies in different climates (Ketzi et al., 2006).

Although FAMACHA has proved to be a practical, effective and popular tool (Leask et al., 2013), the limitations of this system in Europe largely concern the ubiquity of mixed GIN infections and presence of other blood feeding parasites e.g. liver fluke, such that anemia alone cannot reliably reflect impacts on the animal (Di Loria et al., 2009; Papadopoulos et al., 2013). In addition, correlation between the FAMACHA-score and FECs varies from low to high depending on different regions and management systems in Europe (Di Loria et al., 2009; Moors et al., 2009; Scheuerle et al., 2010).

DISCO has been tested for 3 years on several sheep flocks in France and was considered a good indicator (Cabaret, 2004); it was shown to correlate closely with FECs in a study in Morocco (Ouzir et al., 2011).

BODCON is also considered a promising candidate for identifying sheep infected by GIN (Van Wyk and Bath, 2002). Regarding LIVGAIN, animals with low *Teladorsagia* egg counts have higher body weights (Bentounsi et al., 2012) offering the potential through electronic tagging to use automated LIVGAIN as a further diagnostic tool.

#### 5.4.3 Recommendations



The findings of several studies demonstrated that the diagnostic value of FAMACHA, DISCO, BODCON and LIVGAIN in combination with FECs needs to be further investigated in multicenter trials (reviewed in Charlier et al., 2014). Where mixed infections involving multiple genera and species of GIN and other parasites (e.g. liver flukes) are present as in southern Italy (Cringoli et al., 2008, 2009; Rinaldi et al., 2014b), the use of these morbidity parameters is of limited value and direct diagnostic tests (FECs) are mandatory.

## **5.5 PROMOTING FEC/FECR AMONG PRACTITIONERS AND FARMERS**

### *5.5.1. Background*

Promoting FEC/FECR among practitioners and farmers is one of the priority areas for an integrated parasite management where basic and applied research must work together to achieve a sustainable parasite control (Henrioud, 2011). This approach will help parasitologists to better know the real problems of the farmers, detecting new areas of applied research and in turn increase the farmer's awareness (Henrioud, 2011).

Parasitologists, epidemiologists, practitioners and farm advisors should work together to promote practical guidelines for FEC/FECR to sheep farmers. An example is given in UK by SCOPS (Sustainable Control of Parasites in Sheep), a working group formed in 2003 with representatives from the sheep industry to promote practical guidelines for sheep farmers and their advisors. This led to the production of guidelines for veterinarians and sheep advisors, plus promotional literature for farmers (Taylor, 2012) disseminated through the agricultural press, technology transfer events, road-show events and direct communication (McMahon et al., 2013). SCOPS recommendations fall into 2 general categories: i) "Basic Good Practice" using anthelmintics properly and effectively and, i) "Reducing Selection Pressure" avoiding the over-use of anthelmintics, implementing other practices to help reduce the challenge from worms and limiting actions that select heavily for resistance ([www.scops.org](http://www.scops.org)). Amongst the advice promoted by SCOPS, FEC/FECR are actively encouraged.

### *5.5.2. Drawbacks*

Although recommendation to perform FEC/FECR is central to ensuring appropriate anthelmintic control, FEC/FECR are still not widely adopted by veterinary practitioners and sheep farmers (Besier and Love, 2012). Parasitological diagnosis is usually considered a "secondary activity" by the end-users rather than a first step towards a rational guide to GIN

control in small ruminants. Also the costs related to individual faecal sampling and laboratory procedures limit the uptake of FEC/FECR by the end-users (farmer, advisors or veterinarians).

### *5.5.3. Recommendations*

More efforts are needed towards convincing the farmers. The willingness to conduct FEC/FECR will always be driven by the feasibility and compliance by the veterinarians and the farmers. Therefore, a thorough dissemination strategy should be set-up in order to improve communication between parasitologists, practitioners, advisors, farmer associations and farmers. These “door-to-door” or “farm-to-farm” activities are of paramount importance to convince veterinarians and farmers to perform FEC/FECR on a regular basis.

Firstly, there should be clear incentives for both veterinarians and farmers who will be receptive to perform FEC/FECR provided they are convinced of the value and practicality. Evidence of the potential economic losses caused by GIN infection should provide a powerful message regarding the need for effective control programmes (Besier and Love, 2012) and FEC/FECR monitoring will be useful in order to change/adapt parasite control and/or management strategies thus preventing AR. Examples of incentives for veterinarians and farmers could be the delivery of promotional material, with recommendations and guidelines to optimise sustainable control of GIN infections in small ruminants. Also, uploading “vets/farmers corners” on dedicated websites and delivering gadgets (e.g. hats, pens, block-notes, farm clothes, sheep collars, etc.) can be used for promoting parasitological diagnosis.

Secondly, obligations for regular FEC/FECR could be considered, however, this can be more difficult to achieve because stakeholders and politicians should be involved. Stakeholders at local level, farmer associations, and similar organizations should consider the importance of GIN infection as a production disease.

Thirdly, free diagnosis for farmers could be contemplated. An example in Italy is given by the Regional Center for Monitoring Parasitic Infections (CReMoPAR, Campania Region, southern Italy, [www.cremopar.it](http://www.cremopar.it)), coordinated by parasitologists from the University of Naples Federico II (UNINA). CReMoPAR offers free parasitological diagnosis to veterinarians and farmers using (Mini)-FLOTAC techniques for FEC/FECR. Sampling on farms is performed either by the staff at CReMoPAR or by veterinary practitioners during their routine visits on the farms.

However, important issues to consider are the logistical difficulties and costs for FEC/FECR, related to sampling, shipment of faecal samples to diagnostic laboratories and also to the laboratory procedures. Automatization of sampling procedures (e.g. a machine for pooling on the farm) and laboratory techniques for FEC/FECR are the challenge for the next future.

Finally, it is also important that the strategies recommended to farmers are relatively easy to comprehend and that the sampling is easy to perform thus avoiding interference with daily activities on farm.

## **5.6 THE STRATEGY OF MONITORING FEC/FECR IN THE CAMPANIA REGION (SOUTHERN ITALY)**

The strategies/recommendations for FEC/FECR are expected to vary regionally, depending on the local prevalence of the different economically-important parasites, the situation with regard to the efficacy of anthelmintics and AR and regional production systems.

In the Campania region of southern Italy - which extends over an area of 13,590 km<sup>2</sup> and where small ruminant farming has a prominent role for the economy of the region with 9,858 farms and 290,000 animals farmed (10% of the small ruminant livestock of Italy)- an efficient system for promoting FEC/FECR among practitioners and farmers has been established through CReMoPAR since 1995.

Diagnostic, research and dissemination activities are daily ongoing at CReMoPAR. Highly sensitive and accurate diagnostic techniques are used (e.g. FLOTAC, Mini-FLOTAC, Fill-FLOTAC, serology, molecular tools, etc.) and field trials are conducted to study the strategic and economic efficacy of different control strategies against GIN in sheep, goats and other livestock species. Furthermore, spatial epidemiology is used to map and model the distribution of GIN species in small ruminants through the use of modern and powerful resources provided by geographical information systems and other geospatial tools. CReMoPAR is an example of service for livestock that allows academics, veterinarians, and field researchers, to “touch” the real problems of the farmers, detecting new areas of applied research and in turn to increase the farmer’s awareness on the importance of diagnosis and control of GIN infections. CReMoPAR is funded by the Department of Agriculture and

Livestock Production (DALP) of the Campania region and is economically supported by the farmers' associations of Campania and neighboring regions. A huge activity of information with data from research by the parasitologists at UNINA persuaded Officers at DALP and farmers' associations to fund CReMoPAR in consideration of the impact of parasites upon livestock health, welfare and productivity.

The strategies for the management of infections caused by GIN and other parasites infecting sheep in the Campania region of southern Italy are based on ten pillars of paramount importance:

- i) promoting the "Good Practices of Diagnosis" through standardized sampling procedures on farm and standardized FEC techniques in the lab;
- ii) delivering certificates to the veterinarians with the parasitological results to be disseminated to the farmers;
- iii) monitoring GIN infection in sheep farms suggesting at least 3 testings per year;
- iv) advising anthelmintic treatments only when necessary;
- v) recommending the most appropriate anthelmintic drug based on the parasitological results;
- vi) promoting the "Good Practices of Treatment" (correct drenching at the correct dose rate and checking the drug quality);
- vii) monitoring the effectiveness of treatments through FECR;
- viii) promoting targeted treatment based on the epidemiology of GIN in the area;
- ix) performing a "continuing and up-to-date education" on parasitological problems aimed at practitioners, advisors and farmers;
- x) convincing stakeholders on the economic importance of GIN infection as production disease of sheep in order to get funds for diagnosis, research and dissemination activities.

These recommendation and activities are now fairly integrated into routine dairy sheep farm management in the Campania region and, year after year, more and more veterinarians (and sometimes farmers) are bringing faecal samples to the laboratories at CReMoPAR for FEC/FECR. Therefore, after 3-4 days, they receive certificates and advices for treatment by e-mail or *de visu* (if the farm is located nearby CReMoPAR).

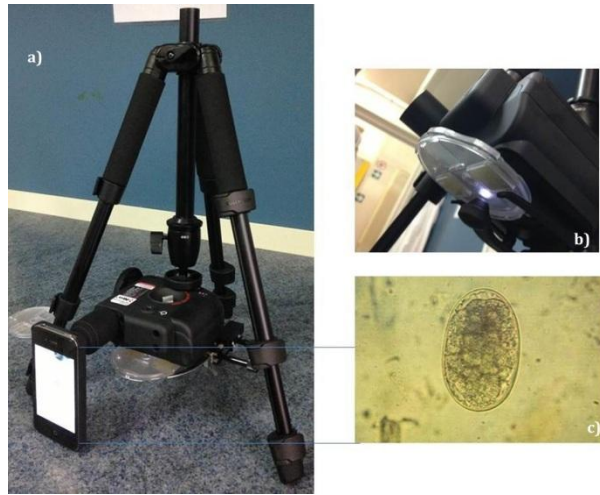
The monitoring of GIN infection in sheep by regular FEC/FECR, the advised use of targeted treatments based on two anthelmintic treatments per year, the rotation of different drugs, the correct drenching, the low movement of sheep between farms, appear to have been effective

in slowing the development of AR in the Campania region of southern Italy (Cringoli et al., 2008; Rinaldi et al., 2014 b).

## **5.7 THE FUTURE OF GIN EGG COUNTS IN SMALL RUMINANTS**

The international economic crisis and the resulting decline of research funds impose the need to resolve issues at considerably lower costs also with respect to diagnosis of GIN in small ruminants taking into account the logistical difficulties in conducting field sampling and the laboratory costs for FEC/FECRT (Cringoli et al., 2013). This is a matter of some importance since the costs and efforts required in undertaking such diagnostic tests may represent a serious impediment to their acceptance and adoption by sheep farmers (Besier and Love, 2012). Hence, now more than ever, to be useful, diagnostic techniques must be accurate, simple and affordable. They must also provide a result in time to institute effective control measures, particularly treatment (Banoo et al., 2010). For these reasons, the adoption of ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable) diagnostic techniques is considered a timely approach in veterinary medicine as well as in public health (Banoo et al., 2010).

Novel solutions are needed to reduce workload/cost for FEC/FECRT; the present thesis provided evidences that a pooled FEC offers cost and logistical advantages for assessing the intensity of GIN in sheep as well as for assessing anthelmintic efficacy (FECR). Together with pooling, one of the challenge of the future of copromicroscopy in livestock is to perform diagnosis of GIN directly on the farm by using field portable kits including the new generation of field microscopes. This approach has been already used with some success in pilot studies in human medicine (Stothard et al., 2005; Bogoch et al., 2013, 2014). Such diagnostic innovations have the benefit of being portable, inexpensive, easy to use, point-of-care tests that do not require a constant electricity supply. Hence, the future of copromicroscopy in small ruminants will depend on the development, standardization and field-evaluation of novel pen-side FEC/FECR tests providing that their results are comparable to those of the well-established laboratory techniques. Commercial and prototype systems are already available (e.g. Field Mini-FLOTAC, FecPakG2, etc.). Such devices (an example is given in Figure 6.1) may not be far from routine on-farm or in epidemiological settings but will require rigorous validation outside of laboratory settings prior to scale-up.



**Fig. 5.1.** Mini-FLOTAC (a) under the Newton NM1 compact portable microscope (note the inverted position) (b). GIN egg (d) visualized by a mobile phone (c) adapted to the portable microscope.

## 5.8. CONCLUSIONS

In the current era of -omics, FEC/FECR have still a future to assess GIN infection intensity and anthelmintic efficacy in small ruminants and other livestock species.

Use of new technologies supported by mobile and electronic (m- and e-health) – based approaches as well as improved and more sensitive strategies of diagnosis are considered one of the priorities towards sustainable solutions to helminth infections in grazing ruminants (Morgan et al., 2013). Now more than ever, veterinary parasitology and public health are converging towards a common strategic approach for optimizing diagnosis of helminths in animals and humans through optimizing FEC/FECR (Mekonnen et al., 2014; Rinaldi and Cringoli, 2014). This thesis outlined some of the challenges in regard to the present assessments and future perspectives of FEC/FECR in small ruminants and identified key areas in which advances in research can help to support effective and efficient strategies against GIN infection for maintaining health, welfare and productivity of small ruminant productions in Europe and beyond.

The research challenges to promote FEC/FECR in the future should be based on: (i) improving existing and/or developing novel FEC/FECR techniques; (ii) optimizing data interpretation towards a sustainable and long-term control program against GIN infections in small

ruminants, and; (iii) develop strategies to convince veterinarians and farmers to perform FEC/FEGR on a regular basis.

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## SUMMARY

Although representing a significant economic burden to the global ruminant livestock industry, infections caused by gastrointestinal nematodes (GIN) in small ruminants are often neglected. Research on these parasites is still lacking, mainly in the matter of diagnostic methods and their use/interpretation. However, the accurate diagnosis and interpretation of GIN infection directly support parasite control strategies, because of the important problems with anthelmintic resistance (AR) in GIN populations of small ruminants. Although various methods can be employed for the *in vivo* diagnosis of GIN infections in small ruminants, faecal egg count (FEC) techniques still remain the most commonly used to assess GIN infections.

In this thesis, the literature review in **Chapter 1** provides an overview of the main FEC techniques used for GIN in small ruminants (McMaster, FECPAK, Wisconsin, FLOTAC and Mini-FLOTAC). Aspects of these FEC techniques are discussed in more detail. Subsequently, we pay special attention to the variability of FECs due to physical (pre-analytic), laboratory (technical) and biological (host-parasite-related) parameters. Finally, we discuss the use and the interpretation of FECs for small ruminants. This review indicates a lack of detailed studies that focus on (i) diagnostic performance of FEC techniques, (ii) factors that influence FECs, and (iii) the final interpretation of these FECs. The overall aim of this thesis is to study the different aspects of the coprological diagnosis of GIN infections in small ruminants with particular emphasis on the significance, interpretation and limitations of FECs.

**Chapter 2** we assessed whether examination of pooled samples provides reliable estimates of the intensity of gastro-intestinal nematode infections (faecal egg counts, FECs) and anthelmintic drug efficacy (faecal egg reduction, FECR). In addition, we verified whether the accuracy of these estimates were affected by pool size and analytic sensitivity of the FEC technique. Ten sheep farms located in Campania in southern Italy were selected for the study. In each farm, individual faecal samples from 20 adult sheep (when possible) were collected, before (D0) and after (D14) an anthelmintic treatment with albendazole. Samples were pooled into pools of 5, 10, and 20 individual samples. Both individual and pooled samples were screened using the FEC technique with an analytic sensitivity of 10 eggs per gram of faeces (EPG, Mini-FLOTAC), 15 EPG (McMaster, McM15) and 50 EPG (McMaster, McM50). GIN FECs of pooled samples correlated positively with mean FECs of individual samples, with very high correlation coefficients (ranging from 0.94 to 0.99) across the 3 different pool sizes and

analytic sensitivities. Mini-FLOTAC was more sensitive compared to the two variants of McMaster (McM15 and McM50) (100% vs 88.5% vs 75.9%) and resulted in significantly higher FEC compared to both McM15 and McM50, with a mean difference in egg counts of approximately 90 EPG ( $P < 0.001$ ). The drug efficacy results showed that FECR was higher than 98% at most farms independently of the pool size and analytic sensitivity. With the exception of two farms, FECR was 100% when calculated for individual animals and across the different pool size and analytic sensitivities. Pooling ovine faecal samples was a rapid procedure that holds promise as a valid strategy for assessing GIN FEC and FECR in sheep.

**Chapter 3** presents the findings of a laboratory trial which compared the degree of measurement error (level of over- or under-estimation of FEC) and precision (variability in FEC) of two FEC methods, Mini-FLOTAC and FecPak techniques, using sheep faeces contaminated with known numbers of nematode eggs. The study showed that the diagnostic performance in terms of measurement error and precision was greater with Mini-FLOTAC. A tendency to under-estimate FEC was observed with the FECPAK particularly at egg densities of less than 500 epg. It is concluded that Mini-FLOTAC is a reliable diagnostic tool offering reduced error and a higher level of precision and accuracy.

**Chapter 4**, a study of GIN egg counts was conducted to improve the accuracy of nematode egg counting in sheep faecal samples. The accuracy and precision of three faecal egg counting (FEC) methods were compared: Mini-FLOTAC combined with Fill-FLOTAC, McMaster and Cornell Wisconsin. Known numbers of eggs extracted from ovine faeces were added to egg free ovine faeces to give counts of 10, 50, 200 and 500 EPG. The Mini-FLOTAC method of counting had the highest sensitivity and accuracy and when combined with Fill-FLOTAC was the best method for counting sheep faecal samples both in the laboratory and on the sheep farm.

In **chapter 5**, the present assessments and future perspectives of FEC/FECR techniques are discussed with particular focus on their application for the detection of AR and as indicator of targeted (selective) treatments. Promoting FEC/FECR among practitioners and farmers is one of the priority areas for an integrated parasite management. However, the costs related to faecal sampling and laboratory procedures limit the uptake of FEC/FECR by these end-users. Novel solutions are needed to reduce workload/cost and to encourage uptake of FEC/FECRT by veterinary practitioners and small ruminant farmers. Together with the strategy of

performing FEC/FECR on pooled faecal samples, one of the challenges of copromicroscopy in small ruminants is to perform diagnosis of GIN directly on the farm by using field portable kits.