THE COPROLOGICAL DIAGNOSIS OF GASTROINTESTINAL NEMATODE INFECTIONS IN SMALL RUMINANTS

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Dissertation submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Veterinary Sciences

2014

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«Ricorda che in ogni attività umana devono esservi tre elementi: in primo luogo le singole imprese devono venir meditate con saggezza prima d’essere realizzate; in secondo luogo devono essere compiute per tempo e con prontezza; in terzo luogo ciò che è stato meditato e compiuto dev’essere serbato e difeso con coraggio».

(Giordano Bruno, Sigillus Sigillorum,1583)

«Remember that in every human activity there must be three elements: firstly, each project must be well thought out before being proposed secondly, it must be completed on time, and thirdly, whatever has been done it should be followed through and defended with courage».

(Giordano Bruno, Sigillus Sigillorum,1583)
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Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many colleagues and friends in Italy and in Belgium.

First and foremost I would like to thank the entire research team of the unit of Parasitology and Parasitic Diseases, Department of Veterinary Medicine and Animal Productions, University of Naples Federico II (Italy), headed by my mentor and “maestro”, Professor Giuseppe Cringoli, a “volcano” of ideas and initiatives. Since 1998, Prof. Cringoli instilled in me the most essential ingredients for successful research: enthusiasm, initiative, ambition, innovation, hard work and intellectual honesty. He is and remains my best role model for a scientist, mentor, and teacher. I would like to thank also my colleagues working at the laboratory of Parasitology in Naples (Doctors Maria Paola Maurelli, Vincenzo Musella, Luisa Del Prete, Emilio Noviello and Paola Pepe) and at the Regional Center for Monitoring Parasitic Infections (Doctors Antonio Bosco, Maria Elena Morgoglione, Mario Parrilla and Mirella Santaniello) for helping me during the field and laboratory studies and for providing support and friendship (…and coffee…. ) that I needed during this hectic period.

I am also extremely grateful to Prof. Jozef Vercruysse and Dr. Bruno Levecke, my supervisors and promoters at the Laboratory of Parasitology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium. I could not have imagined having better advisors and mentors for my PhD. It has been an honor and a privilege for me to work with Prof. Vercruysse, a world leader in veterinary parasitology and a source of inspiration for me. I would like to express my sincere gratitude to him for the continuous support of my research, for his patience, motivation, discipline, enthusiasm, and immense knowledge. I am very proud of what we have achieved together. I am also very grateful to Dr. Levecke for his scientific advice and knowledge and many insightful discussions and suggestions for the success of my PhD. The guidance and patience of Dr. Levecke helped me in all the time of research and writing of this thesis. A special thanks also to Dirk Demeulenaere, Mieke Godefroid and Isabelle Despeghel for the time and efforts they put in helping me. Thank you for your
patience and kindness, for your smiles and sense of humor!

My sincere thanks also to Prof. Gerald Coles and Prof. Eric Morgan from the School of Veterinary Sciences, University of Bristol, for their scientific support in collaborating with me for some experiments that permitted the achievement of my PhD.

I am also very grateful to all the members of the committee of my PhD - Prof. Dr. Frank Jackson, Prof. Christophe Chartier, Prof. Pierre Dorny, Dr. Guy Hendrickx, Dr. Johannes Charlier and Dr. Sofie Piepers - for their careful review of the thesis and for providing me with useful, critical and constructive comments and suggestions.

Finally, I would like to thank my parents (Carlo and Francesca), my sisters (Ida and Simona), all my family and friends (especially Ludovico) for sharing with me this and all the moments of my life. My parents have sacrificed their lives for my sisters and myself and provided unconditional love and care. My friends can always make me laugh...even in serious and challenging periods....

A special thanks to “my favourite parasite”, Giuseppe Iazzetta, for always believing in me and encouraging me to follow my dreams. Thank you for being so lovely!

I very much hope that I shall be able to continue my research, never forgetting to preserve tradition and always contributing to the promotion of the advancement of veterinary parasitology.

Grazie di cuore.

Laura
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAD</td>
<td>Amino Acetonitrile Derivates</td>
</tr>
<tr>
<td>AR</td>
<td>Anthelmintic resistance</td>
</tr>
<tr>
<td>BZ</td>
<td>Benzimidazoles</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CPG</td>
<td>Cysts per gram of faeces</td>
</tr>
<tr>
<td>CREMOPAR</td>
<td>Centro Regionale Monitoraggio Parassitosi (Regional Center for Monitoring of Parasitic Infections), Campania region, southern Italy</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>DALP</td>
<td>Department of Agriculture and Livestock Production of the Campania region (southern Italy)</td>
</tr>
<tr>
<td>DISCONTOOLS</td>
<td>Disease Control Tools</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPG</td>
<td>Eggs per gram of faeces</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FCS</td>
<td>Faecal consistency score</td>
</tr>
<tr>
<td>FEC</td>
<td>Faecal egg count</td>
</tr>
<tr>
<td>FECR</td>
<td>Faecal egg count reduction</td>
</tr>
<tr>
<td>FECRT</td>
<td>Faecal egg count reduction test</td>
</tr>
<tr>
<td>FP</td>
<td>Framework Programme</td>
</tr>
<tr>
<td>FS</td>
<td>Flotation solution</td>
</tr>
<tr>
<td>FS1</td>
<td>Sheather’s sugar solution</td>
</tr>
<tr>
<td>FS2</td>
<td>Satured sodium chloride</td>
</tr>
<tr>
<td>FS3</td>
<td>Zinc sulphate</td>
</tr>
<tr>
<td>FS4</td>
<td>Sodium nitrate</td>
</tr>
<tr>
<td>FS5</td>
<td>Sucrose and potassium iodomercurate</td>
</tr>
<tr>
<td>FS6</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>FS7</td>
<td>Zinc sulphate</td>
</tr>
<tr>
<td>FS8</td>
<td>Potassium iodomercurate</td>
</tr>
<tr>
<td>FS9</td>
<td>Zinc sulphate and potassium iodomercurate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GIN</td>
<td>Gastrointestinal nematodes</td>
</tr>
<tr>
<td>Gl strongyles</td>
<td>Gastrointestinal strongyles</td>
</tr>
<tr>
<td>GLM</td>
<td>Generalized linear model</td>
</tr>
<tr>
<td>GLOWORM</td>
<td>Innovative and sustainable strategies to mitigate the impact of global change on helminth infections in ruminants (FP7) Project KBBE-2011-5-288975</td>
</tr>
<tr>
<td>IVM</td>
<td>Ivermectin</td>
</tr>
<tr>
<td>L1</td>
<td>First-stage larvae</td>
</tr>
<tr>
<td>L2</td>
<td>Second-stage larvae</td>
</tr>
<tr>
<td>L3</td>
<td>Third-stage larvae</td>
</tr>
<tr>
<td>LC</td>
<td>Larval culture</td>
</tr>
<tr>
<td>LCL</td>
<td>Lower confidence limit</td>
</tr>
<tr>
<td>LPG</td>
<td>Larvae per gram of faeces</td>
</tr>
<tr>
<td>LSD</td>
<td>Least significant difference</td>
</tr>
<tr>
<td>LV</td>
<td>Imidazothiazoles/Tetrahydropyrimidines</td>
</tr>
<tr>
<td>McM</td>
<td>McMaster</td>
</tr>
<tr>
<td>ML</td>
<td>Macrocyclic lactones</td>
</tr>
<tr>
<td>MOX</td>
<td>Moxidectin</td>
</tr>
<tr>
<td>MT-PCR</td>
<td>Multiplexed Tandem PCR</td>
</tr>
<tr>
<td>OPG</td>
<td>Oocysts per gram of faeces</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PGE</td>
<td>Parasitic gastroenteritis</td>
</tr>
<tr>
<td>PP</td>
<td>Periparturient period</td>
</tr>
<tr>
<td>PPR</td>
<td>Peri-parturient rise</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SCOPS</td>
<td>Sustainable Control of Parasites in Sheep</td>
</tr>
<tr>
<td>SG</td>
<td>Specific gravity</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedures</td>
</tr>
<tr>
<td>TST</td>
<td>Targeted selective treatment</td>
</tr>
<tr>
<td>TT</td>
<td>Targeted treatment</td>
</tr>
<tr>
<td>UNINA</td>
<td>University of Naples Federico II</td>
</tr>
</tbody>
</table>
1. Location in the host of the prevalent species of GIN infecting small ruminants.

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

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1.4. FECPAK.

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

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<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>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).</td>
</tr>
<tr>
<td>2.</td>
<td>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 - <a href="http://www.gloworm.eu">www.gloworm.eu</a>).</td>
</tr>
<tr>
<td>1.1</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>
(Mini-FLOTAC, McM15 and McM50).

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

5.4. The agreement in FECR across Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).
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 98 million sheep and 16 million goats (FAOSTAT, 2012). 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 DISCONTOOLS 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 99 million € 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.](image)

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).

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

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. Strongylid eggs (70–150 µm) usually hatch within 1–2 days. After hatching, larvae (L1) feed on bacteria and undergo two molts 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.

Temperature and moisture are the dominant factors which influence the free-living stages of *Haemonchus contortus, Teladorsagia circumcincta* and *Trichostrongylus colubriformis*, with the effects of pasture conditions playing a significant modulating role. Early in the free-living phase, the developmental success of the three GIN species is limited by susceptibility to cold temperatures. In general, *H. contortus* is most susceptible, followed by *T. colubriformis* and then *T. circumcincta*. The length of the development cycle is dependent largely on temperature, with development rate increasing at warmer temperatures. However, in order for development to proceed to
the infective larval stage, addition of moisture is generally required. There has been considerably less work quantifying the effects of moisture on free-living development, although it is clear that *H. contortus* is most susceptible to desiccation during the pre-infective stages (O'Connor et al., 2006).

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.](image)

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

### 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.*](image)
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.
V. REFERENCES


CHAPTER 1

Literature review on
“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 (Woolastion, 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, diagnosticians 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 the FLOTAC techniques (Cringoli et al., 2010, 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 copromicroscopy (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 chemicals 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 (Cringoli et al., 2010).

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 eggs in ruminants.

<table>
<thead>
<tr>
<th>Flotation solution</th>
<th>Composition</th>
<th>Specific gravity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose and formaldehyde</td>
<td>C_{12}H_{22}O_{11} 454 g, CH_{2}O solution (40%) 6 ml, H_{2}O 355 ml</td>
<td>1.200</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl 500 g, H_{2}O 1000 ml</td>
<td>1.200</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>ZnSO_{4} \cdot 7H_{2}O 330 g, H_{2}O brought to 1000 ml</td>
<td>1.200</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>NaNO_{3} 315 g, H_{2}O brought to 1000 ml</td>
<td>1.200</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>MgSO_{4} 350 g, H_{2}O brought to 1000 ml</td>
<td>1.280</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>NaNO_{3} 250 g, Na_{2}O_{3}S_{2} \cdot 5 H_{2}O 300 g, H_{2}O brought to 1000 ml</td>
<td>1.300</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>ZnSO_{4} \cdot 7H_{2}O 685 g, H_{2}O 685 ml</td>
<td>1.350</td>
</tr>
<tr>
<td>Sodium chloride and zinc chloride</td>
<td>NaCl 210 g, ZnCl_{2} 220 g, H_{2}O brought to 1000 ml</td>
<td>1.350</td>
</tr>
<tr>
<td>Sucrose and sodium nitrate</td>
<td>C_{12}H_{22}O_{11} 540 g, NaNO_{3} 360 g, H_{2}O brought to 1000 ml</td>
<td>1.350</td>
</tr>
<tr>
<td>Sodium nitrate and sodium thiosulphate</td>
<td>NaNO_{3} 300 g, Na_{2}O_{3}S_{2} \cdot 5 H_{2}O 620 g, H_{2}O 530 ml</td>
<td>1.450</td>
</tr>
<tr>
<td>Sucrose and sodium nitrate and sodium thiosulphate</td>
<td>C_{12}H_{22}O_{11} 1200 g, NaNO_{3} 1280 g, Na_{2}O_{3}S_{2} \cdot 5 H_{2}O 1800 g, H_{2}O 720 ml</td>
<td>1.450</td>
</tr>
</tbody>
</table>
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).

![GIN eggs and Nematodirus egg](image)

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 (µm), shape and shell (data from Thienpont et al., 1986).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Size (µm)</th>
<th>Shape</th>
<th>Shell</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemonchus</em></td>
<td>62-95 x 36-50</td>
<td>Oval; the eggs contain numerous blastomeres hard to distinguish</td>
<td>Thin</td>
</tr>
<tr>
<td><em>Teladorsagia</em></td>
<td>74-105 x 38-60</td>
<td>Oval; the eggs contain numerous blastomeres hard to distinguish</td>
<td>Thin</td>
</tr>
<tr>
<td><em>Trichostrongylus</em></td>
<td>70-125 x 30-55</td>
<td>Oval; the eggs contain 16 to 32 blastomeres</td>
<td>Thin</td>
</tr>
<tr>
<td><em>Cooperia</em></td>
<td>60-95 x 29-44</td>
<td>Oval with parallel sides; the eggs contain numerous blastomeres hard to distinguish</td>
<td>Thin</td>
</tr>
<tr>
<td><em>Nematodirus</em></td>
<td>152-260 x 67-120</td>
<td>Oval; the eggs contain numerous blastomeres hard to distinguish</td>
<td>Thin</td>
</tr>
<tr>
<td><em>Bunostomum</em></td>
<td>75-104 x 45-57</td>
<td>Oval; the eggs contain 4 to 8 blastomeres</td>
<td>Thin</td>
</tr>
<tr>
<td><em>Oesophagostomum</em></td>
<td>65-120 x 40-60</td>
<td>Oval; the eggs contain 16 to 32 blastomeres</td>
<td>Thin</td>
</tr>
<tr>
<td><em>Chabertia</em></td>
<td>77-105 x 45-59</td>
<td>Oval; the eggs contain 16 to 32 blastomeres</td>
<td>Thin</td>
</tr>
</tbody>
</table>

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).
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) 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 diagnostic performance (e.g. analytic sensitivity, precision and accuracy) and technical performance (e.g. ease of use, need for training, cost, safety for user, timing) in assessing FECs. 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. The diagnostic and technical performances of the McMaster, FECPACK, Cornell-Wisconsin, FLOTAC and Mini-FLOTAC techniques are shown in Table 1.3.

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

<table>
<thead>
<tr>
<th>FEC Techniques (amount of faeces used)</th>
<th>Volume (ml)</th>
<th>Reading Area (mm²)</th>
<th>Analytic Sensitivity (Multiplication Factor)</th>
<th>Timing</th>
</tr>
</thead>
<tbody>
<tr>
<td>McMaster (3 to 5 g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 ml</td>
<td>100 mm²</td>
<td>66.6</td>
<td></td>
<td>4 min</td>
</tr>
<tr>
<td>0.30 ml</td>
<td>200 mm²</td>
<td>33.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50 ml</td>
<td>324 mm²</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 ml</td>
<td>648 mm²</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Levecke et al., 2009)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FECPAK (10 g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 ml</td>
<td>216 mm²</td>
<td>20</td>
<td></td>
<td>Less than 10 min</td>
</tr>
<tr>
<td>1.0 ml</td>
<td>432 mm²</td>
<td>10</td>
<td></td>
<td><a href="http://www.texiergroup.co.nz">www.texiergroup.co.nz</a></td>
</tr>
<tr>
<td>1.4 ml</td>
<td>546 mm²</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.8 ml</td>
<td>1092 mm²</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornell-Wisconsin (3-5 g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 ml</td>
<td>324 mm²</td>
<td>1</td>
<td></td>
<td>15-20 min</td>
</tr>
<tr>
<td>(Egwang and Slocombe, 2092)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLOTAC (10 g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ml</td>
<td>648 mm²</td>
<td>1</td>
<td></td>
<td>12-15 min</td>
</tr>
<tr>
<td>(Cringoili et al., 2010)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mini-FLOTAC (5 g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ml</td>
<td>648 mm²</td>
<td>5</td>
<td></td>
<td>10-12 min</td>
</tr>
<tr>
<td>(Bardsa et al., 2013)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Table 1.3. Diagnostic and technical performances of the McMaster, FECPAK, Cornell-Wisconsin, FLOTAC and Mini-FLOTAC techniques.

<table>
<thead>
<tr>
<th>FEC Techniques</th>
<th>Diagnostic performance</th>
<th>Technical performance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Analytic Sensitivity</td>
<td>Precision FEC</td>
</tr>
<tr>
<td>McMaster</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>FECPAK</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Cornell-Wisconsin</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>FLOTAC</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Mini-FLOTAC</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

* The Unit of Parasitology (Department of Veterinary Medicine and Animal Productions, University of Naples Federico II) provides FLOTAC, Mini-FLOTAC and Fill-FLOTAC free of charge to universities, research institutions and other public institutions interested in copromicroscopic diagnosis of parasites.
1.3. PHYSICAL, BIOLOGICAL AND EPIDEMIOLOGICAL FACTORS AFFECTING FECs OF GIN IN SMALL RUMINANTS

A part from the diagnostic and technical performances 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 + (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 adult worm burden and FEC. Some GIN of ruminants undergo arrested development (hypobiosis) at the larval stage in the host, when conditions in the external environment are unfavourable for parasite development and survival (Gibbs, 1986a,b), in which case eggs are not produced by worms and excreted in the faeces (Roeber et al., 2013).

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 EPGs and standard errors) among different individual animals sampled in sheep farms in southern Italy (unpublished data).

Fig. 1.10. Variability of GIN egg counts (mean EPGs 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 a 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.](image)

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 (and worm burden) 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 ≥ 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). 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. 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.

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 the 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 1.4 (adapted from Roeber et al., 2013) summarizes the main principles and limitation of the FECRT.
Table 1.4. Summary of principles and limitations of FECRT (adapted from Roeber et al., 2013).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Principle</th>
<th>Comments and existing limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecal egg count reduction test</td>
<td>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 &lt;95% and the lower confidence limit for the percentage of reduction is below 90%.</td>
<td>- Does not accurately estimate the efficacy of an anthelmintic to remove worms. It rather measures the effects on egg production by mature female worms. Different anthelmintics require sample collection at different time intervals. No agreed standard for FEC method or for the calculation of reduction. Results can be inconclusive due to low analytical sensitivity of the technique. Different results in repeated experiments. Does not provide species specific information. Larval culture required for further differentiation.</td>
</tr>
</tbody>
</table>

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 diagnostic performance (e.g. sensitivity, specificity, precision, accuracy), or technical performance (e.g. simplicity, ease of use, timing, user acceptability, costs) 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.
1.6. REFERENCES


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fecundity of *Teladorsagia circumcincta*. Int. J. Parasitol. 29, 777-780.


APPENDICES

Appendix 1

Simple (gravitational) faecal flotation

1. Homogenize the faecal sample
2. Take a small quantity of faeces (about 3 g)
3. Add flotation solution (about 20 ml)
4. Homogenize the solution with the faeces
5. Filter through a wire mesh (aperture = 250 μm)
6. Transfer the faecal suspension into the tube. Fill to the top and slightly overfill it so that a meniscus forms above the lip of the tube
7. Cover with the coverslip
8. Wait for 10 - 20 min
9. Transfer the coverslip on the glass slide
10. Read

Appendix 2

Faecal Sedimentation

1. Homogenize the faecal sample
2. Mix 100 ml of water with about 10 g of faeces
3. Homogenize thoroughly with a stick
4. Filter through a wire mesh (aperture = 250 μm)
5. The mixture is allowed to sit for 1 hour
6. Remove the 70% of the supernatant
7. Refill the beaker with fresh water
8. Remove the 90% of the supernatant
9. Stir the remaining mixture and place all in a Petri disc or place few drops on the glass slide
10. Read

Repeat steps 5 - 6 - 7 until the suspension is clear
Appendix 3

**Modified McMaster technique**

1. Homogenize the faecal sample
2. Weigh 3 g of fresh faeces
3. Add 42 ml of flotation solution (solution ratio = 1:15)
4. Filter through a wire mesh (aperture = 250 μm)
5. Fill the two flotation chambers
6. Wait for 5 - 10 min
7. Read

Appendix 4

**Wisconsin method**

1. Homogenize the faecal sample
2. Weigh 3-5 g of fresh faeces
3. Add 22 ml of tap water
4. Homogenize thoroughly with a stick
5. Filter through a wire mesh (aperture = 250 μm)
6. Transfer the faecal suspension into the tube
7. Centrifuge 150 x g x 10 min
8. Discard the supernatant
9. Fill the tube (approximately half) with the flotation solution
10. Resuspend the pellet using a wooden stick
11. The tube is topped with FS until a monolayer is formed
12. Place on the top of the tube a 22 x 22 mm coverslip
13. Centrifuge 150 x g x 10 min
14. Transfer the coverslip on the slides
15. Read
Appendix 5

**FLOTAC dual technique**

1. Homogenize and weight 10 g of fresh faeces
2. Add 90 ml of tap water (dilution ratio = 1:10)
3. Homogenize the suspension thoroughly
4. Filter through a wire mesh (aperture = 250 μm)
5. Transfer 2 aliquots, 6 ml each, of the filtered suspension in two conic tubes
6. Centrifuge 170 x g x 3 min
7. Discard the supernatant
8. Fill the tubes with FS to their previous 6 ml levels
9. Fill the two FLOTAC flotation chambers
10. Centrifuge 120 x g x 5 min
11. Translate
12. Read

Appendix 6

**Mini-FLOTAC technique**

1. Homogenize the faecal sample
2. Add 45 ml of flotation solution (dilution ratio = 1:10)
3. Fill the conical collector (5 g of faeces) of the Fill-FLOTAC and level the surface.
4. Homogenize 10 times
5. Fill the Mini-FLOTAC using the filling holes. The flotation chambers are filled with the faecal suspension until a little meniscus is formed
6. Wait for 10 min
7. Translate the top part of the flotation chambers

Read
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) and faecal egg count reduction (FECR) tests.

The specific objectives were:

1. To define the accuracy of the FLOTAC technique and to compare its diagnostic performances with those of other FEC techniques. For this purpose, laboratory trials were conducted on sheep faecal samples to calibrate the FLOTAC compared to simple flotation 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 2].

2. To study the effects of the sampling period (month) and sampling time (hour) on FECs of GIN in small ruminants. For this purpose, a longitudinal study on GIN egg counts was conducted in dairy goats aimed at evaluating: the effect of hour (and month) of faecal sample collection on FECs and the relationship between FECs and adult worm burden [Chapter 3].

3. To evaluate the performance of the FECRT in sheep using the highly sensitive FLOTAC technique and different formulae to calculate anthelmintic efficacy. A further aim was to investigate the efficacy of four different anthelmintics against GIN infections in sheep in a Mediterranean area (southern Italy) [Chapter 4].

4. 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 5].
5. 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 6].
CHAPTER 2

Calibration and diagnostic accuracy of simple flotation, McMaster and FLOTAC for gastrointestinal nematode egg counts in sheep*

### 2.1. INTRODUCTION

Faecal egg count (FEC) techniques are considered relatively straightforward and protocols such as the McMaster technique and the Wisconsin flotation technique have been available for many years (Cringoli et al., 2010).

The different variants of the McMaster method (MAFF, 1986) have the advantage that they are quick to use, particularly if centrifugation is not included in the protocol. For most purposes its sensitivity of 50 or 25 eggs per gram of faeces (EPG) is adequate. However, it is not suitable for situations where sensitive egg counts are required to manage gastrointestinal nematode (GIN) infections and/or for research purposes (Mes et al., 2007; Cringoli et al., 2010; Rinaldi et al., 2010; Levecke et al., 2012a,b). FLOTAC is a multivalent sensitive and accurate copromicroscopic technique of examining faecal samples which allows up to 1 g of faeces to be prepared for microscopic analysis (Cringoli, 2006; Cringoli et al., 2010). Flotation solutions (FS) and faecal preservation methods have a fundamental role in determining the analytic sensitivity (i.e. the smallest amount of parasitic elements in a sample that can be assessed accurately), the precision (i.e. how well repeated observations agree with one another), and the accuracy (i.e. how well the observed values agrees with the ‘true’ values) of any copromicroscopic technique, either qualitative or quantitative, based on flotation, including the FLOTAC techniques (Cringoli et al., 2004, 2010). In view of these considerations, there is a need for detailed calibration of the FLOTAC and other FEC techniques, to determine the optimal FS and faecal preservation method for an accurate FEC of GIN. The present study was aimed at carrying out a calibration and a comparison of diagnostic performance of three FEC techniques, the simple flotation technique (MAFF, 1986), the McMaster (MAFF, 1986) and FLOTAC (Cringoli et al., 2010), in order to find the best FS for GIN egg counts in faecal samples from sheep, and to evaluate the influence of faecal preservation methods combined with FS on egg counts.
2.2. MATERIALS AND METHODS

2.2.1. Calibration of flotation solutions and faecal preservation methods

To determine the optimum FS, faecal preservation method, and technique for counting GIN eggs, faecal samples (60 g) from 10 naturally infected sheep were collected, combined, thoroughly homogenized and divided into four aliquots of 120 g each. These were either directly examined (i.e. fresh), or preserved in 5 or 10% formalin or frozen at −30 °C prior (6-8 days) to counting. Formaldehyde was added at 3 parts fixative to 1 part faeces. To prepare samples for examination by three counting techniques: (i) simple flotation technique (MAFF, 1986), (ii) McMaster technique (MAFF, 1986) and (iii) FLOTAC technique (Cringoli et al., 2010), each aliquot was diluted with 9 parts of water or water plus formalin (i.e. faecal dilution of 1:10), thoroughly homogenised and filtered through a 250 µm wire mesh sieve. The filtered suspension was divided into 162 aliquots of 6 ml to have six replicates for each of 9 FS for the 3 techniques. After centrifugation at 1500 rpm (170 g) for 3 min supernatant was discarded and flotation solutions were added. Tubes were randomly assigned to the three techniques and to the 9 FS described in Table 2.1.
Table 2.1. Flotation solutions used for the calibration of the FEC techniques: chemical composition, specific gravity, toxicity to humans and environment, easiness to prepare, expected storage time and costs.

<table>
<thead>
<tr>
<th>Flotation solutions (chemical composition)</th>
<th>Specific gravity</th>
<th>Toxicity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ease of preparation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Storage time</th>
<th>Costs&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS1 Sucrose and formaldehyde</td>
<td>1.200</td>
<td>E/H</td>
<td>++</td>
<td>1 month</td>
<td>+</td>
</tr>
<tr>
<td>FS2 Saturated Sodium Chloride</td>
<td>1.200</td>
<td>-</td>
<td>+++</td>
<td>Up to 3 month</td>
<td>+</td>
</tr>
<tr>
<td>FS3 Zinc Sulphate</td>
<td>1.200</td>
<td>E</td>
<td>+++</td>
<td>Up to 3 month</td>
<td>+</td>
</tr>
<tr>
<td>FS4 Sodium Nitrate</td>
<td>1.200</td>
<td>-</td>
<td>+++</td>
<td>Up to 3 month</td>
<td>++</td>
</tr>
<tr>
<td>FS5 Sucrose and Potassium Iodomercurate</td>
<td>1.250</td>
<td>E/H</td>
<td>+</td>
<td>1 month</td>
<td>+++</td>
</tr>
<tr>
<td>FS6 Magnesium Sulphate</td>
<td>1.280</td>
<td>-</td>
<td>+++</td>
<td>Up to 3 month</td>
<td>+</td>
</tr>
<tr>
<td>FS7 Zinc Sulphate</td>
<td>1.350</td>
<td>E</td>
<td>+++</td>
<td>Up to 3 month</td>
<td>++</td>
</tr>
<tr>
<td>FS8 Potassium Iodomercurate</td>
<td>1.440</td>
<td>E/H</td>
<td>+</td>
<td>Up to 3 month</td>
<td>+++</td>
</tr>
<tr>
<td>FS9 Zinc Sulphate and Potassium Iodomercurate</td>
<td>1.450</td>
<td>E/H</td>
<td>+</td>
<td>Up to 3 month</td>
<td>+++</td>
</tr>
</tbody>
</table>

Note<br>
<sup>a</sup> E = dangerous for the environment; H = toxic to human; - safe to human and not dangerous for the environment<br>
<sup>b</sup> ++++ very easy; +++ easy; ++ difficult; + very difficult<br>
<sup>c</sup> + very cheap; ++ cheap; +++ expensive; ++++ very expensive

For the simple flotation technique tubes were filled with FS to give a slight meniscus and a 18 mm × 18 mm cover slip was added and left for 15 min before being removed and all eggs counted.

For examination by the McMaster technique (special modification of the McMaster method; MAFF, 1986), FS were added up to 6 ml, the contents of the tube thoroughly mixed and 1.0 ml was then taken up by pipette to load the two cells of the McMaster slide (Weber Scientific International, England; volume = 1.0 ml). Slides were allowed to stand for 10 min before reading both cells. One egg counted was equivalent to 10 eggs per gram of faeces (analytic sensitivity = 10 EPG).

For the FLOTAC technique, FS were added up to 6 ml, the contents thoroughly mixed and used to fill one of the two chambers of the FLOTAC (volume of each chamber = 5 ml). Thus, a single flotation chamber of the FLOTAC was utilized for each replicate
(analytic sensitivity = 2 EPG). After centrifugation of the FLOTAC apparatus at 1000 rpm (120 g) for 5 min, the top of the flotation chambers were translated and the number of eggs counted.

2.2.2. Preservation by vacuum packing

Experiment 2 was aimed at determining the applicability of vacuum packing (Fig. 2.1) as faecal preservation method for GIN egg counts (FEC) by FLOTAC and McMaster (using FS2). Faecal samples (60 g) from 10 naturally infected sheep were collected, combined, thoroughly homogenized and divided into 13 aliquots of 30 g each (see Fig. 2.3). These were either directly examined at day zero (i.e. fresh, D0), or preserved by vacuum packing at room temperature and examined weekly for 28 days (D7, D14, D21, D28), or preserved by vacuum packing in the fridge (+4 °C) and examined weekly for 28 days (D7, D14, D21, D28), or preserved in the fridge (+4 °C) without vacuum packing and examined weekly for 28 days (D7, D14, D21, D28). Vacuum packing was performed using a domestic appliance; this method can be used for preserving samples (van Wyk, J. personal communication).

Fig. 2.1. Vacuum packing used for preservation of sheep faecal samples.
2.2.3. **Statistical analysis**

The arithmetic mean eggs per gram of faeces (EPG), standard deviation (SD), and Coefficient of Variation (CV) of EPG values were calculated for the different FS for each preservation method and each technique. CV \([\text{standard deviation/mean}]*100\) was calculated for the 6 replicates of each flotation solution (i.e. intra-assay CV). Differences between flotation solutions, techniques and preservation methods were analyzed using an one-way ANOVA with post hoc Fisher’s least significant difference (LSD). Statistical analysis was carried out using STATA 10.0 software (Stata Corp., TX 77845, USA). In addition, a likelihood ratio test of the equality of the CV of k normally distributed populations was performed using a software developed by the Statistical Services at the Forest Products Laboratory (USA; http://www1.fpl.fs.fed.us/covtestk.html).

2.3. **RESULTS**

2.3.1. **Calibration of flotation solutions and faecal preservation methods**

Figure 2.2 which shows GIN egg counts (EPG and CV) in the composite sheep faecal sample, stratified by diagnostic technique, FS and faecal preservation method. The “gold standard” FS was defined as the FS which produced the highest EPG and the lowest CV. Statistical comparisons were performed only for FS producing EPG above the 50% of the gold standard (marked with a blue line in Fig. 2.2).

Both the FLOTAC and McMaster techniques gave acceptable counts on fresh faeces with all the solutions from FS1 to FS6 but usually with a lower CV with FLOTAC counts. The “gold standard” for GIN egg counts was obtained with FLOTAC when using FS5 (EPG = 320, CV = 4%) and FS2 (EPG = 298, CV = 5%). In the samples examined fresh with FLOTAC, the CV of FS5 was significantly lower \((P < 0.05)\) than the CV resulted when using FS1, FS7 and FS9. No significant difference \((P > 0.05)\) was found between the CV of FS5 and the CVs of FS2, FS3, FS4, FS6, FS8.

None of the methods of preservation (using formalin or freezing) provided satisfactory results for GIN egg counts. Moreover, the results from simple flotation were unacceptably low.
Fig. 2.2. The recovery of eggs of GIN from sheep faeces by FLOTAC, McMaster and tube flotation using 9 different flotation solutions and 4 different methods of sample preservation. *P <0.05; significant differences for different letters.
2.3.2. Preservation by vacuum packing

Anaerobic storage by using vacuum packing of faecal samples and refrigeration at 4 °C permitted GIN egg counts up to 21 days after collection although after this time some egg structure began to disappear (Fig. 2.3). Mould formed by day 14 if refrigeration without vacuum packing was used limiting the acceptability of this method. Preservation by vacuum packing with storage at room temperature (21.8 °C) was satisfactory until day 28 but from day 7 the smell from the samples limited their acceptability. From day 14 there was an increase in larvation of the eggs of GIN.

Fig. 2.3. GIN egg counts (EPG) using McMaster or FLOTAC counts in fresh faecal samples or stored by refrigeration, vacuum packing or refrigeration and vacuum packing for up to 28 days (D7, D14, D21, D28).
2.4. DISCUSSION

If a simple fast decision is required on whether sheep should be treated for infection with GIN the present data confirms that the McMaster technique or its on farm version, FECPAK (Presland et al., 2005), are satisfactory, although the best results, in terms of sensitivity, accuracy and precision was obtained with the FLOTAC technique, as in previous studies (Cringoli et al., 2010). The simple flotation technique should never be used due to the very low and variable results obtained. Instead, although not used in the present study, flotation in centrifuge, i.e. the Wisconsin and modified Cornell-Wisconsin techniques (Egwang and Slocombe, 1981, 1982) are known to be highly sensitive methods for recovering GIN eggs when in low numbers in cattle faeces. The lower sensitivity, accuracy and precision of the McMaster technique for egg counts has been also mentioned by Mes et al. (2007), who have reported that this technique requires extrapolation, and thus it renders EPG estimates less precise than methods that do not require extrapolation, such as the Wisconsin flotation method (Cox and Todd, 1962; Egwang and Slocombe, 1982) and the modified salt–sugar flotation method (Mes et al., 2007). Following this line of thought, larger multiplication factors are needed for extrapolation, for example, under the smaller McMaster slide areas (volumes), the less precise EPG counts will result. Moreover, using the FLOTAC technique, a large amount of faecal suspension is examined, and so also the sensitivity is greater; thus, this technique is less likely to give false negative results (Rinaldi et al., 2010). The results confirm, also, that the faecal preservation methods and the flotation solutions have a fundamental role in determining the analytic sensitivity of any copromicroscopic technique; it is noteworthy if a sample has examined to be fresh, it does not produce the same results if the method of faeces preservation changes (e.g., frozen, fixed in formalin or in other fixatives). Freezing and chemical preservation (formaldehyde) should not be used for storage of GIN eggs as demonstrated also by Foreyt (1986).

Vacuum storage or refrigeration can be used to store faecal samples for up to 21 days without significantly reducing the egg counts, although the best combination is both vacuum packing and refrigeration, and so it could be a good alternative method to preserve the faeces to analyze and should be tried also on other helminth eggs of other animal species. Detailed studies on the ecology (threshold of temperature and moisture) of GIN eggs could be of interest to find the most appropriate method to stop egg development when storing faecal samples.
With respect to the choice of FS, both the FLOTAC and McMaster techniques gave acceptable counts on fresh faeces with all the solutions from FS1 to FS6 but usually with a lower CV with FLOTAC counts. The “gold standard” for GIN egg counts was obtained with FLOTAC when using FS5 (sucrose and potassium iodomercurate) and FS2 (saturated sodium chloride). However, the toxicity of the chemicals (mercury salts) contained in the FS5 and the strict legal requirements for their disposal will exclude its use for FECs (Cringoli et al., 2010). Hence, based on the results of the present calibration study on GIN egg counts, we suggest the use of saturated sodium chloride (FS2), a solution that is easy and cheap to purchase and prepare and that is safe for human and not dangerous for the environment.
2.5 REFERENCES


CHAPTER 3

Is gastrointestinal nematode faecal egg count influenced by hour (and month) of sample collection and adult worm burden in goats?*

3.1. INTRODUCTION

Infection by gastrointestinal nematodes (GIN) is of particular economic importance in goat production systems worldwide (Rinaldi et al., 2007b). The study of host-parasite relationships towards a sustainable control of these parasites usually requires reliable estimates of parasite intensity, which is often estimated from faecal egg counts (FECs). However, parasite excretion in faeces may be subject to variation due to endogenous or exogenous factors that must be identified to obtain reliable results (Villanua et al., 2006). Concerning the biological factors, FEC may be subjected to a great within-individual variation due to factors such as host reproductive status, weather, season, random day-to-day variation, and the phase of the parasitic infection (Villanua et al., 2006). In order to study the factors that can influence the significance of FEC results, the present paper reports a longitudinal study on GIN egg counts (FECs) in dairy goats aimed at evaluating: (i) the effect of hour (and month) of faecal sample collection on GIN egg counts and (ii) the relationship between FECs and adult worm burdens.

3.2. MATERIALS AND METHODS

3.2.1. Study farm and study animals

The study was conducted at the experimental farm of the “C.R.A., Unità di Ricerca Zootecnia Estensiva, Bella Scalo, Muro Lucano”, located in the Potenza province of southern Italy at 360 m above sea level. A total of 63 female Siriana goats (unmated) were used for the study. They were approximately 1.5 years old and in their second grazing season. The goats in the farm grazed for 8 h/day and were supplemented with concentrates, corresponding to 50% of energy requirements. These goats, together with the rest of the flock, had been treated with moxidectin in June. Before the start of the study (July 2005) each goat was randomly assigned to a sampling day (see below).

3.2.2. Relationship between the hour and the month of sampling and GIN egg counts

Every 3 weeks - from 13th July 2005 to 6th September 2006 (total = 21 weeks) - faecal
samples were collected every 2 h for 24 h from three of the goats. At each sampling day, three goats were individually housed in digestibility cages containing a sieve in the bottom for separating faeces, as described by Fedele et al. (2002). The three cages were placed in the box where the rest of the flock was housed during the night after grazing. It should be noted that in order to avoid possible bias due to the caging, the experimental goats were acclimatized to the cages each evening for 1 week before each sampling day. Goats were fed with hay and concentrates when caged. In addition, a soft lamp illuminated the stable so that goats were not disturbed by the technicians during the faecal sample collection. Thus, in the present study we attempted to avoid any confounding factor, being aware, however, that the change of diet resulting from grazing pasture to being confined in the box may have influenced the results. Every 3 weeks for 14 months faecal samples were collected every 2 h for 24 h from the individually caged goats. The faeces were those passed by the goats during the 2 h preceding each collection. For each goat a 10 g sample was obtained from this material, thoroughly homogenized, and then analyzed using the FLOTAC double technique (Cringoli, 2006; Cringoli et al., 2010) having an analytic sensitivity of 2 eggs per gram of faeces (EPG). To explore relationships between FECs and adult parasite counts, on the day following sampling, the three goats were euthanized and the nematodes present in the abomasa and intestines were recovered, identified and counted.

**3.2.3. Relationship between adult worm burden and FECs**

On each sampling day, in order to have FECs representative of the 24 h for each goat, a 5 g pooled sample from the 12 individual samples was analyzed using the FLOTAC double technique as described above. In addition, on each sampling day a composite faecal culture was made per goat (MAFF, 1986). On the day following faecal sampling, the three goats were euthanized, and adult nematodes in the abomasa and intestines were recovered, identified and counted (Wood et al., 1995). The detailed procedures used for faecal culture and determining worm burden are described below.

**3.2.3.1. Larval culture**

The faeces were broken up finely using a large spatula. If faeces were very dry they were damped with water; if the faeces were very wet, then sterile faeces were added until the
correct consistency was obtained. Wide-mouthed jars were then filled with the mixture, the lid replaced, and incubated at 27 °C for 7 days. Larvae were then recovered using the Baermann apparatus (MAFF, 1986).

Third stage larvae were identified using the morphological keys proposed by Gevrey (1971), Lancaster and Hong (1987) and van Wyk et al. (2004). When a coproculture had 100 or less third stage larvae, all were identified; when more than 100 larvae were present, only the first 100 were identified. Noteworthy, Teladorsagia and Trichostrongylus larvae were difficult to be differentiated based on sheath extension length alone. To further refine their differentiation, additional morphological features were required based on the presence of an inflexion at the cranial extremity of Teladorsagia larvae (Roeber et al., 2013).

3.2.3.2. Worm burden

The viscera were processed for sample collection, further worm counts and identification of adult parasites present in the abomasum and small and large intestines, following the procedures described in the WAAVP guidelines for evaluating the efficacy of anthelmintics in ruminants (Wood et al., 1995).

Double ligatures were placed at the omasal and pyloric ends of the abomasum, as well as the ileocecal junction. The abomasum, small and large intestines were separated, freed of excess fat and mesenteric attachments (Wood et al., 2005).

**Abomasal content** - The abomasum was opened and its content was thoroughly washed into a graduated bucket under a slow running jet of tap water. The volume of washed content was made up to 2 liters. After thorough mixing, two 5% aliquots were withdrawn from the bucket and mixed with sufficient 10% formalin for later examination (Wood et al., 2005).

**Abomasal mucosa** - The mucosal surface was scraped from the abomasum. The digestive solution used was constituted by 1% pepsin and 3% HCl. The volume, by weight, of this solution was at least three times the weight of the mucosa. The mucosal material was digested in the digestive solution and in a water bath at 37-40°C for no longer than 4-6 h. The digest was poured through a 38/µm sieve. The residue was diluted with tap water to 2 liters, the suspension was thoroughly mixed and two 5% aliquots were fixed in formalin for later examination.

**Small intestine** - The small intestines were opened along their entire length and
Chapter 3

their contents were directed into and collected in a large container. The opened intestines were rinsed twice and then squeezed through the fingers with the contents being directed into the same container. The volume of washed contents was made up to 2 liters. After thorough mixing, two 5% aliquots were withdrawn from the bucket and were mixed with sufficient 10% formalin for later examination (Wood et al., 2005).

Large intestine - The contents of the large intestine were collected and processed in the same way as for the small intestine. However, large adult worms were detached from the mucosal surface and the contents were washed over a large sieve (150 μm) before worms were retrieved and counted (Wood et al., 2005).

Recovering, counting and identifying nematodes - The samples from the abomasum and intestines were passed through a 38 μm sieve and the residue were transferred into wide mouthed jars (Wood et al., 2005). The parasites in a 5% aliquot from the abomasal and intestinal contents and the abomasal mucosa were counted. The numbers, species and stages of nematodes in each aliquot from each animal were recorded. Few drops of 45% iodine solution were added to a sample to stain the nematodes and the background was cleared with 5% sodium thiosulfate. For each aliquot, adult nematodes were removed and transferred to microscope slides for detailed examination. A drop or two of lactophenol were added to destain the adult nematodes in order to facilitate the identification (Wood et al., 2005).

3.2.4 Statistical analysis

Mean, standard error and 25th, 50th and 75th percentiles of GIN EPG values (not ln-transformed) were calculated for each sampling hour interval, pooling the data from the 63 goats. Then, EPG values were ln-transformed in order to achieve the Normal distribution, as detected by the analyses of the Normality tests of Shapiro–Wilk ($P >0.05$) and the Normal Q–Q Plots. On these ln-transformed data a generalized linear model (GLM) for the analysis of the effect of hour (and month) of sample collection on GIN egg counts was performed. In particular, EPG values were introduced in the model as dependent variables, hour and month of sampling as categorical fixed factors and individual animal was entered in the model as a random factor.

Mean and standard error of females, males and EPG counts were calculated for each GIN
species based on pooling data from the 63 goats. The degree of aggregation of FECs and worm counts of GIN was assessed using the parameter k from the negative binominal distribution. Adult worm count values and EPG were ln-transformed in order to achieve the Normal distribution, as detected by the analyses of the Normality tests of Shapiro–Wilk ($P >0.05$) and the Normal Q–Q Plots. On these ln-transformed data, for each GIN species, the relationship between adult worm burden and EPG was evaluated using Pearson correlation. All the statistical analyses were performed using SPSS software (Version 13) and STATA 9.2 software.

### 3.3. RESULTS

#### 3.3.1. Relationship between the hour and the month of sampling and GIN egg counts

Table 1 shows the GIN EPG values (arithmetic means) every 2 h over the 24-h sampling period, pooled for the 63 goats. The k values indicate that the data for all EPGs were aggregated (see below). The mean values of GIN EPG ranged from 4417.0 (hour interval 2.00–4.00) to 8652.9 (hour interval 6.00–8.00). The results of GLM (controlling for the effect of individual by considering it as a random effect) did not reveal any significant effect of the hour of sample collection ($F_{11,63} = 0.99; P = 0.449$) on FECs, whereas a significant effect of the sampling month (seasonality) was found ($F_{20,63} = 27.5; P = 0.000$). Figure 3.1 shows the FEC values during all the study period; the highest EPG values were observed between April and June. Data on monthly temperature (Tmin, Tmean, Tmax) in the study area are also reported in Figure 3.1, whereas data on rainfall were not available. However, the association between the environmental variables and the monthly EPG trend was not investigated in the present study.
Table 3.1. Two-hourly GIN EPGs (arithmetic means) over 24 h pooled for 63 goats.

<table>
<thead>
<tr>
<th>Sampling time (2-hour interval)</th>
<th>Arithmetic mean</th>
<th>Standard error</th>
<th>Percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>25\textsuperscript{th}</td>
</tr>
<tr>
<td>8.00-10.00</td>
<td>7254.2</td>
<td>1800.7</td>
<td>1943</td>
</tr>
<tr>
<td>10.00-12.00</td>
<td>6184.2</td>
<td>1006.4</td>
<td>1883</td>
</tr>
<tr>
<td>12.00-14.00</td>
<td>4838.3</td>
<td>965.9</td>
<td>143</td>
</tr>
<tr>
<td>14.00-16.00</td>
<td>4947.4</td>
<td>866.3</td>
<td>1658</td>
</tr>
<tr>
<td>16.00-18.00</td>
<td>6923.2</td>
<td>1537.3</td>
<td>1974</td>
</tr>
<tr>
<td>18.00-20.00</td>
<td>6816.9</td>
<td>1316.8</td>
<td>1890</td>
</tr>
<tr>
<td>20.00-22.00</td>
<td>5686.3</td>
<td>989.8</td>
<td>1904</td>
</tr>
<tr>
<td>22.00-24.00</td>
<td>4449.3</td>
<td>793.9</td>
<td>1012</td>
</tr>
<tr>
<td>24.00-2.00</td>
<td>6965.3</td>
<td>1426.2</td>
<td>1025</td>
</tr>
<tr>
<td>2.00-4.00</td>
<td>4417.0</td>
<td>855.4</td>
<td>1163</td>
</tr>
<tr>
<td>4.00-6.00</td>
<td>8025.1</td>
<td>1572.4</td>
<td>1634</td>
</tr>
<tr>
<td>6.00-8.00</td>
<td>8652.9</td>
<td>1605.8</td>
<td>2185</td>
</tr>
</tbody>
</table>

Fig 3.1. Monthly GIN EPGs (arithmetic means) in the studied goats and temperature trend (Tmin, Tmean, Tmax) in the study months.
3.3.2. Relationship between adult worm burden and FECs

The adult nematodes recovered and identified in the goats were: *Teladorsagia circumcincta* and *Haemonchus contortus* in the abomasum; *Trichostrongylus colubriformis* in the small intestine; and *Oesophagostomum venulosum* in the large intestine. It should be noted that during the study no inhibited larvae (hypobiotic larvae) were observed in all the animals sampled.

GIN EPG and adult worm population were aggregated with a positive skewed distribution; this aggregation was also found among the four species of GIN, especially *H. contortus* (see the values of the k parameters from the negative binomial distribution in Table 3.2). Table 3.2 also shows the arithmetic mean and standard error of the different species of GIN (EPG, number of females, males and total worms), the female/male ratio and the correlation between EPG and total number of adult per each species of GIN (data pooled for 63 goats). The mean number of adult GIN counted in the 63 studied goats was 4447.2 (range = 310–13,992).
Table 3.2. GIN egg counts (FEC) and adult parasite counts (arithmetic means) pooled for 63 goats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>GIN</th>
<th>Haemonchus contortus</th>
<th>Oesophagostomum venulosum</th>
<th>Trichostrongylus colubriformis</th>
<th>Teladorsagia circumcincta</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPG Mean</td>
<td>7660.8</td>
<td>3866.7</td>
<td>345.4</td>
<td>2490.5</td>
<td>958.2</td>
</tr>
<tr>
<td>Standard error</td>
<td>1287.7</td>
<td>753.3</td>
<td>79.2</td>
<td>406.9</td>
<td>146.2</td>
</tr>
<tr>
<td>k parameter*</td>
<td>0.65</td>
<td>0.70</td>
<td>0.70</td>
<td>0.70</td>
<td>0.68</td>
</tr>
<tr>
<td>Females Mean</td>
<td>2844.4</td>
<td>555.4</td>
<td>17.5</td>
<td>1725.9</td>
<td>545.6</td>
</tr>
<tr>
<td>Standard error</td>
<td>280.6</td>
<td>87.5</td>
<td>3.2</td>
<td>215.1</td>
<td>77.5</td>
</tr>
<tr>
<td>k parameter*</td>
<td>0.66</td>
<td>0.71</td>
<td>0.72</td>
<td>0.71</td>
<td>0.68</td>
</tr>
<tr>
<td>Males Mean</td>
<td>1602.8</td>
<td>410.8</td>
<td>13.6</td>
<td>851.6</td>
<td>326.8</td>
</tr>
<tr>
<td>Standard error</td>
<td>154.3</td>
<td>61.8</td>
<td>2.8</td>
<td>111.9</td>
<td>39.2</td>
</tr>
<tr>
<td>k parameter*</td>
<td>0.66</td>
<td>0.71</td>
<td>0.72</td>
<td>0.71</td>
<td>0.66</td>
</tr>
<tr>
<td>Female/male ratio</td>
<td>1.7</td>
<td>1.3</td>
<td>1.3</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Total worms (females+males)</td>
<td>Mean</td>
<td>4447.2</td>
<td>966.2</td>
<td>31.1</td>
<td>2577.5</td>
</tr>
<tr>
<td>Standard error</td>
<td>431.1</td>
<td>148.2</td>
<td>5.6</td>
<td>323.0</td>
<td>115.3</td>
</tr>
<tr>
<td>k parameter*</td>
<td>0.66</td>
<td>0.71</td>
<td>0.72</td>
<td>0.71</td>
<td>0.68</td>
</tr>
<tr>
<td>Pearson correlation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P&lt;0.001)</td>
<td>0.619</td>
<td>0.915</td>
<td>0.728</td>
<td>0.501</td>
<td>0.404</td>
</tr>
</tbody>
</table>

* Negative binomial parameter (P<0.001).
The most prevalent GIN species was *T. colubriformis*, followed by *H. contortus*, *T. circumcincta* and *O. venulosum*. The mean EPG in the 63 studied goats was 7660.8 (min 100, max 52,330). Combining the results of FECs and coprocultures, *H. contortus* showed the highest egg output, followed by *T. colubriformis*, *T. circumcincta* and *O. venulosum*. The scatter plots of FEC and adult worm burden for each GIN species are reported in Figure 3.2. The Pearson correlation results showed a positive relationship between FECs and total GIN adult worm burden (*r* = 0.619; *P* < 0.001). At species level, the highest positive relationship was found for *H. contortus* (*r* = 0.915; *P* < 0.001), followed by *O. venulosum* (*r* = 0.728; *P* < 0.001), *T. colubriformis* (*r* = 0.501; *P* < 0.001), and *T. circumcincta* (*r* = 0.404; *P* < 0.001).
Fig. 3.2. Scattered plots of GIN egg counts (EPG) and adult worm burden (ln-transformed data) for each GIN species.
3.4. DISCUSSION

This study demonstrated no evidence of a circadian rhythm in the FECs of GIN in goats. The study did show, however, a significant relationship between FECs and adult parasite counts on consecutive days, especially for *H. contortus*. The lack of a significant effect of the hour of sample collection on FECs was also reported by Bennett (1990) for strongyle parasites of equines, whereas a circadian rhythm of egg excretion has been observed for other nematode species, e.g. *Heligmosomoides polygyrus* in wild wood mice (Brown et al., 1994) and *Passalurus ambiguus* in rabbits (Rinaldi et al., 2007a). The findings of the present study have important practical implications, since they demonstrated for the first time that faecal sampling for GIN egg counts (FECs) can be performed at any moment of the day on a goat farm without affecting FEC values. These results can be likely extended to sheep and cattle farms, since all these ruminants often share the same parasitic genera and/or species. In the present study, as expected, FECs were affected by month of sampling, and this should be considered in the design of parasite control programs for goats in regions with similar climate and management (Veneziano et al., 2004; Cringoli et al., 2008). The FEC and adult worm burden results of the present study showed that most of the GIN burden may occur in a small percentage of hosts. Indeed, the distribution of FECs (at any hour of sample collection) and adults (both females and males) was asymmetrically positive. These findings are in agreement with other studies on small ruminants (Barger, 1985; Cabaret et al., 1998; Hoste et al., 2001, 2002). Secondly, the Pearson correlation results of the present study showed a positive relationship between FECs and total GIN adult worm burden ($r = 0.6$). This positive relationship was found for all the GIN species. A positive correlation between EPG and GIN adult counts has been previously found in dairy goats from several temperate and steppe areas, in particular when *H. contortus*, the most prolific species, was present (McKenna, 1985; Cabaret and Gasnier, 1994; Cabaret et al., 1998). In addition, Roberts and Swan (1982) also found a strong correlation between FECs and the total number of *H. contortus* adults in naturally infected sheep and, more recently, Beriajaya and Copeman (2006) demonstrated a strong relationship between FECs and adult worm burden in sheep experimentally infected by *T. colubriformis*. It is interesting that in our study the correlation between species egg counts (estimated from the coproculture findings) and adult worm burden followed the pattern of fecundity of the different
species of GIN, hence suggesting that there are different host/parasites influences on per capita egg output.

This relationship between FECs and adult worm burden may be influenced by many factors such as fecundity of species, age of worm, volume of ingesta and host resistance (Roberts and Swan, 1982). However, the association between FECs and adult worm burden is not an illusion, even considering density-dependant phenomenon (Cabaret et al., 1998). The female–male ratio was similar to that observed in other studies (Coadwell and Ward, 1982; Paolini et al., 2003; Good et al., 2006). The strong linkage between FECs and adult worm burden supports the use of FEC techniques to measure the prevalence and intensity of infections for epidemiological surveys, to quantify the efficacy of treatments, and to detect anthelmintic resistance (Eysker and Ploeger, 2000). In conclusion, the present study showed that the hour of sample collection does not influence the GIN FECs in goats and that there is a good relationship between FECs and total GIN adult worm burden in goats. Gathering of this kind of information is recommended as an initial step for any host–parasite study (Villanua et al., 2006), and further research is needed on the influence of hour of sample collection and relationship with worm burden for other parasites of goats and for other ruminant species.
3.5. REFERENCES


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Parasitol. 113, 253-261.


CHAPTER 4

Faecal Egg Count Reduction Test:
use of FLOTAC and different formulae to evaluate anthelmintic efficacy

INTRODUCTION

Anthelmintic resistance (AR) has become an urgent global issue in the control of gastrointestinal nematodes (GIN) of sheep and goats in major small ruminant producing regions, such as South America, Australia, South Africa and the UK, with multiple AR found on many farms (Jackson and Coop, 2000; Kenyon et al., 2009; Kaplan and Vidyashankar, 2012). A recent study performed in France, Greece and Italy (Geurden et al., 2014) showed that AR against (pro-)benzimidazoles and levamisole is widespread in sheep in these EU countries (with differences between countries and farms) and that the efficacy of moxidectin and ivermectin was still high on the selected farms.

There are several conventional methods for the detection of AR in sheep, including both in vitro and in vivo tests (Coles et al., 1992, 2006; reviewed in Demeler et al., 2012). Among these latter in vivo tests, also methods for the molecular diagnosis of AR in sheep nematodes have been developed but only for the analysis of benzimidazole resistance (Demeler et al., 2012).

In vitro tests generally involve incubation of free-living parasite stages (eggs or larvae) of GIN in a range of drug concentrations followed by measurement of vitality in form of development, motility or migration. Currently, five main assays are used, including (i) egg hatch test, (ii) larval development, (iii) motility, (iv) migration and (v) feeding assay. We will not discuss the procedures of these assays in more detail, instead we would like to refer the reader to Demeler et al. (2012).

In vivo tests include the (i) worm reduction test and (ii) the faecal egg count reduction test (FECRT). For the worm reduction test, animals are necropsied at the end of the trial, after which the remaining worms in the intestinal tract of the treated animals are compared with those from animals that did not receive any treatment. For the FECRT, the change in egg excretion after treatment is compared, depending on the study design, with either those before treatment of the same animal or with those from animals that did not receive any treatment (Coles et al., 1992, 2006).

Up to date the FECRT is the only method that allows to assess drug efficacy of all anthelmintics (vs. the in vitro tests and molecular methods) against all GIN species (vs. in vitro tests) and without sacrificing the animals (vs. worm reduction test) (McKenna, 2013). Guidelines on how to perform a FECRT are made available by the World Association for the Advancement of Veterinary Parasitology (WAAVP). These guidelines
(Coles et al., 1992) provide recommendations on the experimental set up (randomized control trial), sample size (≥10 or ≥15 animals per treatment group, with a mean EPG greater than 150), laboratory technique (McMaster, analytic sensitivity of 10 to 50 EPG), statistical analysis (FECRT based on the arithmetic mean of grouped FECs after drug administration) and criteria defining reduced efficacy (FECRT <90% or FECRT <95% and lower limit of 95% confidence interval <90%).

Since the publication of these WAAVP guidelines, an increasing number of issues have arisen. First, the randomized controlled study design advocated in the WAAVP guidelines was less sensitive at detecting AR compared to FECRT based on pre- and post-treatment counts from the same animals (McKenna, 2006; Dobson et al., 2012; Calvete and Uriarte, 2013). Second, the poor detection limit (10 to 50 EPG) of the recommended FEC method may thwart the precision of FECRT results in cases of low baseline FECs (El-Abdellati et al., 2010; Levecke et al., 2011; Torgerson et al., 2012; Calvete and Uriarte, 2013). Alternative newly developed FEC methods, (Mini-) FLOTAC (Cringoli et al., 2010, 2013) and FECPAK (www.fecpak.com) can be used, allowing for the detection of up to 1 and 5 eggs per gram of faeces (EPG), respectively.

Recently, various formulae to calculate FECRT have been advocated (individual vs. group based and geometric vs. arithmetic mean) and uncertainty exists on which formula to apply (Vercruysse et al., 2001; Cabaret and Berrag, 2004; Dobson et al., 2009, Levecke et al., 2011; Vercruysse et al., 2011; Calvete and Uriarte, 2013).

The aim of the present study was therefore to evaluate the performance of FECRT in sheep using the highly sensitive FLOTAC technique (Cringoli et al., 2010) and different formulae to calculate anthelmintic efficacy. A further aim was to investigate the efficacy of four different drugs against GIN infections in sheep in a Mediterranean area (southern Italy).
4.2. MATERIALS AND METHODS

4.2.1. Study area

The study was conducted in the Campania region of southern Italy. In this area, sheep farms are widely distributed with an average area of approximately 50 ha. The area is mainly used for cereal production but small pastures occur on upland areas that are unsuitable for cropping (Fig. 4.1).

Fig. 4.1. Study area.
4.2.2. Study farms and animals

Trials were conducted between 2008 and 2011 on 27 sheep farms. Dairy sheep farms were randomly selected throughout the region and the selection was mainly driven by the willingness of the farmer (27 out of 50 sheep farmers contacted gave positive consent). The animals used for the trials were mainly local regional breeds, e.g. Bagnolese (for milk) and dairy crossbreeds (e.g. Comisana x Sarda) (Fig. 4.2). These animals were kept on the pasture all year round.

![Experimental animals](image)

Fig. 4.2. Experimental animals.

On each farm all animals were weighed and given the correct dose. Noteworthy, with ivermectin a half dose was also included to indicate whether AR to the macrocyclic lactones might be developing (Palmer et al., 2000). Tests were run with groups of sheep (12 to 20 animals per group) using four anthelmintics administered orally, namely: levamisole (Levacide, Norbrook, 7.5 mg/kg) on 8 farms, ivermectin (Oramec, Merial, 0.1 and 0.2 mg/kg) on 8 farms, moxidectin (Cydectin, Pfizer, 0.2 mg/kg) on 3 farms and monepantel (Zolvix, Novartis, 2.5 mg/kg) on 8 farms. It should be noted that moxidectin was used only in 3 farms because there was an outbreak of brucellosis in the area were the other 5 farms were supposed to be included in the study. Faecal samples were collected rectally on days 0 and 7 for levamisole and monepantel (Hosking et al., 2009; Jones et al., 2010) and on days 0 and 14 for ivermectin and moxidectin. The anthelmintic classes, drugs and dose as well as the number of farms and animals used in the study are given in Table 4.1.
### Table 4.1. The anthelmintics used on sheep farms in southern Italy.

<table>
<thead>
<tr>
<th>Anthelmintic class</th>
<th>Anthelmintic drug</th>
<th>Dosage of drug (mg/kg)</th>
<th>No. of sheep farms tested</th>
<th>No. of animals per treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM</td>
<td>Levamisole</td>
<td>7.5</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>ML</td>
<td>Ivermectin</td>
<td>0.2</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ivermectin</td>
<td>0.1</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Moxidectin</td>
<td>0.2</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>AAD</td>
<td>Monepantel</td>
<td>2.5</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

IM = Imidazothiazoles; ML = Macrocyclic lactones; AAD = Amino Acetonitrile Derivates.

### 4.2.3. Laboratory procedures

Individual faecal egg counts were determined using the FLOTAC double technique (Cringoli et al., 2010) with a sensitivity of 2 eggs per gram (EPG) of faeces, using a sodium chloride based flotation solution (FS2, specific gravity = 1.200). In addition, on each sampling day a composite faecal culture was conducted for each group (MAFF, 1986). Third stage larvae were identified to the level of genus using the morphological keys proposed by van Wyk et al. (2004). When a coproculture had 100 or fewer third stage larvae, all were identified; when more than 100 larvae were present, only the first 100 examined were identified.

### 4.2.4. Statistical analysis

On each faecal sampling occasion, arithmetic mean EPG was calculated as recommended by the WAAVP guidelines for evaluating the efficacy of anthelmintics in ruminants and, for each treatment group, percent efficacy (%) was calculated in terms of FECR on the different days (Coles at al., 1992; McKenna et al., 2006). Specifically, the following formulae were used:

1) \[
\text{FECR1} = 100 \times (1-[T2/T1][C1/C2]),
\]

where \(T1\) and \(T2\) represent the mean pre- and post-treatment FECs of the treated group, respectively, and \(C1\) and \(C2\) represent the mean pre- and post-treatment FECs of the untreated control group, respectively (Dash et al., 1988; Pook et al., 2002; Dobson et al., 2009). Confidence intervals were estimated by bootstrapping. Thus, pre- and post-treatment individual FECs for T and C groups
were simulated from the original values with replacement, and FECR re-calculated. The procedure was repeated 10,000 times, and the 2.5 and 97.5 percentiles of the simulated results were used as 95% CI. The PopTools (CSIRO, Australia) add-in to Excel (Microsoft Corp, USA) was used for these simulations.

2) \( \text{FECR}_2 = 100 \times (1-[T_2/C_2]) \), where \( T_2 \) represents the mean post-treatment FECs of the treated group, and \( C_2 \) represents the mean post-treatment FECs of an untreated control group (Coles et al., 1992). Arithmetic means were used and 95% Confidence Intervals (CI) calculated using variance in treatment and control groups as set out in Coles et al. (1992).

3) \( \text{FECR}_3 = 100 \times (1-[T_2/T_1]) \), where \( T_1 \) and \( T_2 \) represent the mean pre- and post-treatment FECs of each treated group, respectively (Dobson et al., 2012). Confidence intervals were estimated by bootstrapping as described for FECR1.

As suggested in the WAAVP guidelines (Coles et al., 1992), the following criterion was used for defining reduced efficacy: \( \text{FECR} < 95\% \) and lower limit of 95% confidence interval <90%.

4.3. RESULTS

The efficacies of the anthelmintic treatments are given in Tables 4.2 to 4.5, for levamisole, ivermectin, moxidectin and monepantel, respectively. Very high efficacy (FECR ≥98%) was obtained with all anthelmintics, independent of the FECR formula used. For levamisole the mean FECR between farms equalled 99.3% (range 98-100%). For the macrocyclic lactones, the FECR equalled 99.5% (98.0-100%), 99.9% (99.3-100%) and 100% (99.9-100%) for ivermectin half dose, ivermectin full dose and moxidectin, respectively. For monepantel, the mean FECR was 99.4% (97-100%).

Lower confidence limits (LCL) were generally high and always above 95% for monepantel and 99% for moxidectin. On two of the eight farms on which ivermectin was used, LCL when using a half dose was between 90 and 95% using one or more statistical methods; for the full dose, the minimum LCL was 95.5% using FECR1 and 97.8% using FECR2. For levamisole, two of the eight farms tested showed LCL below 95%, using one or both methods. Based on the values of FECR (>95%) and LCL (>90%), the efficacy of all the drugs tested were classified as “normal” regardless formulae and the presence of
control group.
The genera of nematodes present (minimum and maximum percentages in each treatment group) at the time of treatment were: *Trichostrongylus* (68.9-80.4%); *Teladorsagia* (11.6-16.3%); *Oesophagostomum/Chabertia* (2.7-11.2%); *Haemonchus* (1.9-7.4%); *Cooperia* (0.6-2.6%) and *Bunostomum* (0-0.2%). There was no significant variation of these percentages in relation to year, whereas some variation was found in relation to the period of sampling, especially regarding *Teladorsagia* that showed the highest prevalence in autumn (unpublished data).

Table 4.2. Mean GIN egg counts (EPG) at day 0 and at day 7 and activity of levamisole at 7.5 mg/kg in naturally infected sheep in southern Italy, calculated by three formulae (FECR1, FECR2 and FECR3).

<table>
<thead>
<tr>
<th>Farm number</th>
<th>Treatment</th>
<th>EPG at day 0</th>
<th>EPG at day 7</th>
<th>FECR1 (%)</th>
<th>FECR2 (%)</th>
<th>FECR3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>95% CI</td>
<td>95% CI</td>
<td>95% CI</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>183</td>
<td>151</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Levamisole</td>
<td>108</td>
<td>2</td>
<td>98.3</td>
<td>99.0</td>
<td>98.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(92.0 – 100)</td>
<td>(94.8 - 99.8)</td>
<td>(97.4 – 99.3)</td>
</tr>
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Table 4.3. Mean GIN egg counts (EPG) at day 0 and at day 7 and activity of ivermectin at 0.1 mg/kg (IVM1) and at 0.2 mg/kg (IVM2) in naturally infected sheep in southern Italy, calculated by three formulae (FECR1, FECR2 and FECR3).

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Table 4.4. Mean GIN egg counts (EPG) at day 0 and at day 7 and activity (FECR) of moxidectin at 0.2 mg/kg in naturally infected sheep in southern Italy, calculated by three formulae (FECR1, FECR2 and FECR3).

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Table 4.5. Mean GIN egg counts (EPG) at day 0 and at day 7 and activity (FECR) of monepantel at 2.5 mg/kg naturally infected sheep in southern Italy, calculated by three formulae (FECR1, FECR2 and FECR3).

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4.4. DISCUSSION

The findings of the present survey showed a very high anthelmintic efficacy in sheep farms in southern Italy for all the drugs used and regardless the formula employed for calculation of FECR (based on the presence/absence of a control group). Mean values of FECR (%) were above 98% for all the anthelmintics tested, namely levamisole, ivermectin (half and full dose), moxidectin and monepantel. Also lower confidence limits (LCL) were generally high and always above 90% for all the anthelmintics regardless the statistical method used to calculate FECR. Therefore, according to the WAAVP guidelines (Coles et al., 1992), we did not detect reduced efficacy (i.e. FECR <95% and LCL <90%) in any of the tested sheep farm. A detailed examination of the mean FECR and LCL provided by the three different formulae (FECR1, FECR2, FECR3) showed that the use of either procedure was likely to result in similar estimates. As a consequence, in cases where FECR is near 100%, as in the present survey, the more complex and costly FECR1 and FECR2 (involving the control groups) are unlikely to provide any real advantages over the simpler FECR3 method. In addition, the presence of a control group could also represent a bias for FECRT if randomization is not properly performed and if there are differences in mean EPG between the control and treated group at baseline (Torgerson et al., 2005) as occurred in some farms of the present study. However, it should be underlined that the agreement among the different formulae may change in case of low FECR (reduced efficacy or anthelmintic resistance) and this may affect the interpretation of FECRT. Anyway, the possibility of using an easy method for FECRT (without control groups) is a matter of some importance since the costs and effort required in undertaking FECRT may represent a serious impediment to their acceptance and adoption by sheep farmers (Besier and Love, 2012). It should be noted that, when using different drugs on the same farm, a further abbreviated version of the FECR3 can be used, i.e. FECR4 \( (FECR4 = 100 \times (1 - T2/C1)) \) where \( C1 \) represented the arithmetic mean of a single pre-treatment group (McKenna, 2013, 2014).

The accuracy of the FECRT is often considered dependent on the sensitivity of the technique used for faecal egg counting (Demeler et al., 2012; Levecke et al., 2012 a,b). The findings of our survey suggest that the percentage of FECR for deciding whether anthelmintic resistant nematodes are present in sheep could be raised when using high sensitive techniques as FLOTAC (Cringoli et al., 2010). This would enable a small reduction in efficacy to be detected and this will be particularly valuable with
anthelmintics which have a very high efficacy against GIN of sheep with FECR between 98 and 99%, as ML’s (Cringoli et al., 2008, 2009) and monepantel (Hosking et al., 2009; Jones et al., 2010), thus permitting early detection of anthelmintic resistance which is crucial to avoid exponential increase of AR and associated production losses.

The main limitation of our findings was represented by the high efficacy (FECR = 100% in most of the farms) of anthelmintics found in the present study; it is likely that differences in calculation and results would have possibly occurred in farms with different levels of AR. 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). In contrast, in southern Australia, a region having a Mediterranean climate similar to that of southern Italy, the prevalence and extent of AR to all classes of broad-spectrum anthelmintics is so widespread that it threatens the profitability of the entire sheep industry (Besier and Love, 2003). The uncommon AR in sheep in southern Italy could be explained by the low treatment frequency and therefore the occurrence of a significant refugia within the hosts. Furthermore, also refugia on the pastures are very abundant and they are not destroyed by the summer dryness due to a favorable microclimate in some zones of the pasturing areas. Although a constant monitoring of the efficacy of anthelmintics in sheep in southern Italy is strongly recommended (Charlier et al., 2014), the FECRT remains a time and labour intensive test, particularly when control groups are included (in such a case formulae FECR1 and FECR2 are used). Concerning the sensitivity of laboratory techniques, although highly sensitive techniques as FLOTAC (up to 1 EPG) could be useful to detect low levels of AR, it should be underlined that it requires centrifuging and thus the GIN egg counts have to be lab based. An alternative could be represented by the introduction of Mini-FLOTAC (Cringoli et al., 2013) that is sensitive up to 5 EPG. In addition, to further reduce the cost of resistance testing for farmers the use of pooled faecal samples (Morgan et al., 2005) would be advised to reduce workload/cost and to encourage uptake of the FECRT by veterinary practitioners and farmers.
4.5. REFERENCES


Hosking, B.C., Griffiths, T.M., Woodgate, R.G., Besier, R.B., Le Feuvre, A.S., Nilonm, P.,


CHAPTER 5

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

5.1. INTRODUCTION

The accurate diagnosis of gastrointestinal 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, studies on the effect of pool size have been conducted in goats, for GIN (Cabaret et al., 1986) and coccidia (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. 5.1) 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. 5.1. Mini-FLOTAC.](image)

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.

5.2. MATERIALS AND METHODS

5.2.1. 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. 5.2) on the farms were naturally infected with GIN (*Trichostrongylus* spp., *Haemonchus contortus* and *Teladorsagia circumcincta*) (Dipinetto 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. 5.2. Experimental animals.](image)

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 5.3.

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 5.4. 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. Each pooled sample was thoroughly homogenized by stirring with a plastic tongue spatula by two different laboratory assistants for at least 1 min each. As for the individual samples, each pool was examined using McMaster and Mini-FLOTAC.
5.2.2. Parasitological examination

5.2.2.1. 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 µm) 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 GIN, were obtained by multiplying the total number of eggs by 50 (McM50) or 15 (McM15).
5.2.2.2. **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.

5.2.3. **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 described below.

5.2.3.1. **Comparison of individual and pooled samples for assessment of FEC and drug efficacy (FECR)**

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 FECR using the formula below:

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

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.

5.2.3.2. Agreement in qualitative and quantitative diagnosis of GIN across FEC techniques

The three copromicroscopic techniques (Mini-FLOTAC, McM15 and McM50) were compared qualitatively (sensitivity) and quantitatively (FECs).

A positive sample was defined if positive with any parasitological method, while a negative sample was considered negative if negative with all methods. As the diagnostic “gold standard”, we considered the combined results from McMaster methods plus Mini-FLOTAC method. 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.

5.2.3.3. Agreement in assessment of anthelminthic drug efficacy (FECR) across FEC techniques

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.
5.3. RESULTS

5.3.1. Comparison of individual and pooled samples for assessment of FEC and FECR

5.3.1.1. 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. ≥ 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).

![Concordance plots for different copromicroscopic techniques](image)

Fig. 5.5. 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 5.1. 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 5.1. 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).

<table>
<thead>
<tr>
<th>Pair-wise comparison</th>
<th>Mean difference in FECs (EPG)</th>
<th>(P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mini-FLOTAC</td>
<td>McM15</td>
</tr>
<tr>
<td>Individual vs pools of 5</td>
<td>90</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>(0.27)</td>
<td>(0.10)</td>
</tr>
<tr>
<td>Individual vs pools of 10</td>
<td>86</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>(0.30)</td>
<td>(0.05)</td>
</tr>
<tr>
<td>Individual vs pools of 20</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>(0.96)</td>
<td>(0.68)</td>
</tr>
</tbody>
</table>

5.3.1.2. **Agreement in assessment of FECR**

Table 5.2 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 pre-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).
5.3.2. Comparison of diagnosis and assessment of drug efficacy across FEC methods

5.3.2.1. 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. 5.6. 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.](image)
Table 5.2. The agreement in FECR across different pool sizes (N) and copromicroscopic techniques (Mini-FLOTAC, McM15 and McM50).

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>Mini-FLOTAC</th>
<th>McM15</th>
<th>McM50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ind N = 5</td>
<td>N = 10</td>
<td>N = 20</td>
</tr>
<tr>
<td></td>
<td>FECR (%)</td>
<td>FECR (%)</td>
<td>FECR (%)</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>99.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>98.8</td>
<td>97.3</td>
<td>99.1</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5 to 10</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Ind = individual samples*
Table 5.3 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 5.3. The agreement in FECs across Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

<table>
<thead>
<tr>
<th>Pair-wise comparison</th>
<th>Pearson correlation coefficient (( P )-value)</th>
<th>Mean difference in FECs (( P )-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini-FLOTAC vs McM15</td>
<td>0.98 (&lt;0.001)</td>
<td>90.9 (&lt;0.001)</td>
</tr>
<tr>
<td>Mini-FLOTAC vs McM50</td>
<td>0.97 (&lt;0.001)</td>
<td>87.0 (&lt;0.001)</td>
</tr>
<tr>
<td>McM15 vs McM50</td>
<td>0.99 (&lt;0.001)</td>
<td>-3.9 (0.98)</td>
</tr>
</tbody>
</table>

5.3.2.2. **Agreement in assessment of anthelmintic drug efficacy (FECR)**

Table 5.4 summarizes per farm the number of animals included in the efficacy trial, mean FECs at baseline, FECR 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 FECR 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 FECR results no attempts were taken to verify correlation, and differences in FECR and the final interpretation between the three techniques.
Table 5.4. The agreement in FECR across Mini-FLOTAC and the two variants of the McMaster method (McM15 and McM50).

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>No. samples</th>
<th>Mini-FLOTAC</th>
<th>McM15</th>
<th>McM50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean FEC at D0</td>
<td>FECR (95%CI)</td>
<td>Mean FEC at D0</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>1396</td>
<td>100(99.9;100)</td>
<td>999</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>261</td>
<td>99.6(98.7;100)</td>
<td>173</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>536</td>
<td>98.8(97.5;99.5)</td>
<td>341</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>52</td>
<td>100(100;100)</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>1830</td>
<td>100(100;100)</td>
<td>1444</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>225</td>
<td>100(100;100)</td>
<td>219</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>4078</td>
<td>100(100;100)</td>
<td>3599</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>3621</td>
<td>100(100;100)</td>
<td>3428</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>360</td>
<td>100(100;100)</td>
<td>333</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>72</td>
<td>100(100;100)</td>
<td>54</td>
</tr>
</tbody>
</table>
5.4. 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 (≥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., 2014). 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). However, in the present study, a preliminary estimation gave a cost-benefit of 44 Euros for a FEC on pooled samples (n = 20) compared to the examination of 20 individual samples. This calculation was performed considering the following parameters: a) 12 Euros is the hourly pay rates of a diagnosticians in Italy; b) processing and reading a Mini-FLOTAC requires approximately 12 min (Barda et al., 2013); pooling samples requires some additional time (5 minutes for 20 samples).
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 WAAVP (Coles et al., 1992) 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 for assessing the intensity of GIN (FEC) in sheep as well as anthelmintic efficacy (FECR). However, further research is required (i) to optimize and standardize the methodology of pooling faecal samples; and (ii) to verify the validity of the pooled FECR test also in settings where AR is present.
5.5. REFERENCES


CHAPTER 6
Overall Discussion

Gastrointestinal nematode faecal egg counts in small ruminants: present assessments and future perspectives
6.1. 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., 2014a). 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 calibration and performance of different FEC techniques and the effect of different faecal preservation methods on GIN egg counts; ii) the effects of the sampling time (hour) and period (month) on FECs of GIN in small ruminants; iii) the performance of FECRT in small ruminants using a high sensitive FEC technique (FLOTAC) and different formulae to calculate efficacy of anthelmintics, and; iv) the value of pooled faecal samples to assess GIN infection intensity (FEC) and anthelmintic efficacy (FECR) in sheep.

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 5 highlighted that: i) FLOTAC, saturated sodium chloride, and vacuum packing at +4°C are the most accurate FEC technique, flotation solution and preservation method, respectively, for reliable GIN egg counts in sheep (Chapter 2, Rinaldi et al., 2011); ii) the hour of faecal sample collection does not influence GIN egg counts and there is a good relationship between FECs and total GIN worm burden in small ruminants (Chapter 3, Rinaldi et al., 2009); iii) easy methods for FECRT (without control groups) can be used in surveys aimed at detecting anthelmintic resistance in cases where FECR is near 100%, as in the Campania region of southern Italy where a very high anthelmintic efficacy was detected in sheep farms for all the drugs, regardless of the formula to calculate FECR (Chapter 4, Rinaldi et al., 2014b); iv) 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 5, Rinaldi et al., 2014a). In the next paragraphs we will discuss the importance of a “continuing and up-to-date education” on FEC/FECR to parasitologists, diagnosticians, 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.

6.2. THE ROLE OF FEC/FECR FOR THE DETECTION OF ANTHELMINTIC RESISTANCE

6.2.1. 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., 2014a). 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.

6.2.2. 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.

6.2.3. 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.

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

#### 6.3.1. 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 FECs, weight gain, body condition score, and milk yield (reviewed in Charlier et al., 2014b). 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.
6.3.2. 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 ≥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). 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).

6.3.3. 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., 2014a). 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).

6.4. NEED FOR OTHER DIAGNOSTIC TOOLS IN COMBINATION WITH FEC

6.4.1. 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).

6.4.2. 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, Teladorsagia, Trichostrongylus* or *Oesophagostomum*. However, GIN posses 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 (Ketzis 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 (Ouizir 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.

6.4.3. 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., 2014a). 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.

6.5. PROMOTING FEC/FECR AMONG PRACTITIONERS AND FARMERS

6.5.1. 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, ii) “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). Amongst the advice promoted by SCOPS, FEC/FECR are actively encouraged.

6.5.2. 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).

6.5.3. 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), 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. Because the farmer is the primary decision-maker concerning the control of helminths, the farm level is likely to be suited best to the improvement of helminth disease management (Charlier et al., 2014b). More efforts are needed to providing the short and long term cost benefits of promoting diagnosis since this is crucial in persuading diagnosticians, veterinarians and farmers to adopt these approaches.

6.6. 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 km² 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. Then, 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).

6.7. 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. 6.1. 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.
6.8. 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) developing strategies to convince veterinarians and farmers to perform FEC/FECR on a regular basis.
6.9. REFERENCES


McKenna, P.B., 2013. Are multiple pre-treatment groups necessary or unwarranted in


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 assesses the accuracy of three FEC techniques (McMaster, FLOTAC and simple flotation) for GIN faecal egg counts (FEC). In addition we evaluated the impact of flotation solutions (FSs, 9 different solutions) and preservation of samples (fresh, vaccum packed, formalin (5% and 10%) and freezing) on FECs. Overall, FLOTAC resulted in similar or higher FECs compared to McMaster. Simple flotation underestimated the FECs. FLOTAC was the most accurate method (lowest coefficient of variation (CV)). The best FS for FLOTAC were those based on sucrose plus potassium iodomercurate (FEC = 320 eggs per gram of faeces (EPG), CV = 4%) and saturated sodium chloride (FEC = 298 EPG, CV = 5%). Vacuum packing with storage at +4°C permitted storage of GIN eggs for up to 21 days prior to counting. Freezing and preservation in formalin (5% and 10%) resulted in underestimating the original FECs.
In chapter 3, a longitudinal study of GIN egg counts was conducted on 63 naturally infected dairy goats to evaluate the effects of the hour (and month) of sample collection on FECs and the relationship between FECs and adult worm burden. Every 3 weeks for 14 months, faeces were collected every 2 hours for 24 hours from three individually caged goats. For each goat, individual FECs were performed using the FLOTAC technique with an analytic sensitivity of 2 eggs per gram of faeces. Subsequently, the three goats were euthanized and the adult nematodes in the abomasa and intestines were counted and identified. The results indicated no significant difference in FECs within 24h, but they indicated a significant difference across the 14 months. There was a significant correlation between FECs and worm burden, in particular regarding *H. contortus*.

In chapter 4, a field study was conducted to evaluate the efficacy of anthelminthic drugs against GIN in small ruminants by means of three Faecal Egg Count Reduction (FECR) formulae. These formulae mainly differ whether a control group was included. The efficacy was evaluated for 4 anthelminthic drugs in 27 sheep farms in southern Italy, including levamisole (8 farms), ivermectin (half and full dose, 8 farms), moxidectin (3 farms) and monepantel (8 farms). FECRT were run with groups of 12 to 20 sheep. Faecal samples were collected on days 0 and 7 for levamisole and monepantel, and on days 0 and 14 for ivermectin and moxidectin. Individual FECs were determined using the FLOTAC technique with a sensitivity of 2 eggs per gram faeces. A reduced efficacy was claimed when FECR was <95% and the lower limit of 95% confidence interval (LCL) <90%. The results showed a very high anthelmintic efficacy in sheep farms in southern Italy for all the drugs, regardless of the formula to calculate FECR. Mean values of FECR (%) were above 99% for all the drugs tested. In addition, LCLs were generally high and always above 90% for all the anthelminthic drugs regardless of the formula.

In chapter 5, we assessed whether examination of pooled samples provides reliable estimates of the intensity of gastrointestinal nematode infections (faecal egg counts, FECs) and anthelminthic 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 techniques 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.

In chapter 6, 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.
Ondanks hun economische impact op de wereldwijde veeteeltindustrie, krijgen gastro-intestinale nematoden (GIN) bij kleine herbekkers onvoldoende wetenschappelijke aandacht, vooral op het vlak van diagnose. Een nauwkeurige diagnose gevolgd door een correcte interpretatie van de resultaten vormt echter de basis voor het uitwerken van controlestrategieën, omdat resistentie tegen ontwormingsmiddelen voor GIN infecties wereldwijd een probleem vormt bij kleine herbekkers. Er zijn verschillende technieken voor het *in vivo* opsporen van GIN in kleine herbekkers, maar coprologische technieken op basis van het tellen van eieren uitgescheiden in de mest, de zogenaamde ‘faecal egg counts’ (FEC), worden het meest toegepast om zowel worminfecties als de efficiëntie van ontwormingsmiddelen te evalueren.

In **Hoofdstuk 1** wordt een overzicht gegeven van de belangrijke FEC technieken voor het opsporen van GIN infecties bij kleine herbekkers (McMaster, FECPAK, Wisconsin, FLOTAC en Mini-FLOTAC). We bespreken de operationele aspecten van deze technieken in detail. Vervolgens schenken we aandacht aan de variatie in eitellingen veroorzaakt door fysische, technische (in het laboratorium) en biologische (interactie tussen worm en gastheer) factoren. Ten slotte bespreken we hoe deze eitellingen gebruikt en geïnterpreteerd worden voor kleine herbekkers. Deze literatuurstudie toont aan dat, hoewel FEC technieken beschouwd worden als de diagnostische standaardprocedure, er een tekort is aan gedetailleerde studies die focussen op (i) de diagnostische performantie van FEC technieken, (ii) factoren die eitelling beïnvloeden, en (iii) de uiteindelijke interpretatie van deze eitellingen. Dit proefschrift focust daarom op verschillende aspecten van coprologische diagnose van GIN infecties bij kleine herbekkers, met bijzondere aandacht voor de betekenis, de interpretatie en de beperkingen van eitellingen in mest.

In **Hoofdstuk 2** wordt de nauwkeurigheid van drie belangrijke FEC technieken voor eitellingen bij GIN onderzocht, namelijk McMaster, FLOTAC en eenvoudige flotatie. Eitellingen op basis van FLOTAC waren vergelijkbaar of hoger dan die van McMaster. Eenvoudige flotatie onderschatte steeds de eitellingen. FLOTAC was het nauwkeurigst (kleinste variatiecoëfficiënt (VC)). Aanvullend wordt ook de impact van de flotatievloeistof (9 verschillende vloeistoffen) en de manier van bewaren (geen bewaring, vacuüm verpakt, in formaline (5% en 10%) en invriezen). De meeste optimale
flotatievloeistoffen voor FLOTAC waren deze op basis van sucrose and kalium iodomercurate (FEC = 320 EPG, VC = 4%) en verzadigde natriumchloride oplossing (FEC = 298 EPG, VC = 5%). Vacuümverpakking aan 4°C tot 21 dagen gaf vergelijkbare eitellingen met de oorspronkelijke bij verse mest. Invriezen en formaline (5% en 10%) resulteerde in een onderschatting van de originele tellingen.

In **Hoofdstuk 3** werd aan de hand van een longitudinale studie in 63 geiten nagegaan of het tijdstip van bemonsteren een impact heeft op de eitelling en in welke mate eitelling een betrouwbare inschatting geeft van het aantal wormen. Meststalen werden verzameld van 3 individueel gehuisveste dieren gedurende 24 uur elke 2 uur. Alle meststalen werden onderzocht met de FLOTAC techniek (analytische sensitiviteit van 2 eieren per gram mest). Vervolgens werden de dieren geëutanaseerd en werden het aantal wormen in het gastro-intestinaal stelsel geteld en geïdentificeerd. Dit proces werd om de 3 weken herhaald gedurende 14 maanden. Er was geen significant verschil in eitellingen over de 24 uur, maar wel over de 14 maanden. Er was een significante correlatie tussen eitelling en het aantal wormen (r = 0.6, p = 0.000), dit in het bijzonder voor *H. contortus* (r = 0.9).

In **Hoofdstuk 4** werd de efficiëntie van ontwormingsmiddelen tegen GIN in kleine herkauwers berekend aan de hand van 3 verschillende formules voor de reductie in ei-uitscheiding (faecal egg count reduction, FECR). Deze formules variëren hoofdzakelijk in het al dan niet gebruik van een onbehandelde controlegroep. De efficiëntie van 4 ontwormingsmiddelen (levamisole, ivermectine, moxidectine en monepantel) werd geëvalueerd in 27 schapenbedrijven in Zuid-Italië, namelijk levamisole (8 bedrijven), ivermectine (halve en volledige dosis, in 8 bedrijven), moxidectine (3 bedrijven) en monepantel (8 bedrijven). De FECR werd uitgevoerd op groepen van 12 tot 20 schapen. Meststalen werden verzameld op dag 0 en 7 voor levamisole en monepantel en op dag 0 en 14 voor ivermectine en moxidectine. Alle stalen werden onderzocht met de FLOTAC (analytische sensitiviteit van 2 eieren per gram mest). Een ontwormingsmiddel was niet efficiënt als de FECR <95% lag en als de ondergrens van het 95% betrouwbaarheidsinterval <90 % lag. Alle gebruikte geneesmiddelen hadden een zeer hoge efficiëntie ongeacht de FECRT formule. De gemiddelde waarden van FECR lagen boven 99 % voor alle geteste ontwormingsmiddelen. Ook de ondergrens was over het
algemeen hoog en altijd boven de 90 % voor alle ontwormingsmiddelen, ongeacht de FECR formule.

In **Hoofdstuk 5** werd nagegaan of het onderzoek van mengstalen betrouwbare schattingen geeft van de worminfectiegraad en de efficiëntie van ontwormingsmiddelen in schapen. Aanvullend werd nagegaan of deze schattingen beïnvloed worden door het aantal gemengde stalen en door de analytische sensitiviteit van de diagnostische methode. Er werd een veldstudie uitgevoerd in 10 schapenbedrijven in Campanië (Zuid-Italië). Elk bedrijf verzamelde (waar mogelijk) meststalen van 20 volwassen schapen vóór (dag 0) en na (dag 14) behandeling met albendazole. Voor mengstalen werden 5, 10 en 20 individuele stalen gemengd. Zowel individuele als mengstalen werden onderzocht met een analytische sensitiviteit van 10 EPG (Mini-FLOTAC), 15 EPG (McMaster, McM15) and 50 EPG (McMaster, McM50). De worminfectiegraad op basis van mengstalen correleerde in hoge mate met deze van individuele stalen (0,94-0,99) en dit voor elke mengstaalgrootte en analytische gevoeligheid. De Mini-FLOTAC was echter gevoeliger voor het opsporen van worminfecties (100 % versus 88,5 % (McM15) versus 75,9 % (McM50)). Ook de eitellingen waren significant hoger ten opzichte van zowel McM15 als McM50. Gemiddeld was het verschil in eitellingen 90 eieren per gram mest (P <0,001). De efficiëntie van de behandeling was hoger dan 98% voor de meeste bedrijven, en dit ongeacht de mengstaalgrootte en de analytische gevoeligheid. Met uitzondering van twee boerderijen was de efficiëntie van de ontwormingsmiddelen 100 %, berekend voor individuele dieren en over de verschillende mengstaalgroottes en analytische gevoeligheden. De resultaten tonen aan dat het mengen van stalen een betrouwbare schatting oplevert van zowel worminfectiegraad als efficiëntie van ontwormingsmiddelen bij schapen.

In **Hoofdstuk 6** wordt het gebruik van FEC technieken besproken met bijzondere aandacht voor de toepassing op de detectie van AR en als indicator van controlestrategieën. Aanvullend volgen suggesties voor het promoten van FEC/FECR onder dierenartsen en veehouders als een van de prioriteiten van een geïntegreerd wormmanagement. De kosten voor bemonstering en laboratoriumprocedures beperken echter de uitvoering van FEC/FECR door deze eindgebruikers. Nieuwe oplossingen zijn nodig om de werklast en de kosten te verminderen en het gebruik van FEC/FECRT te
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bevorderen bij dierenartsen en boeren van kleine herkauwers. Samen met de strategie van het uitvoeren van FEC/FECR op gepoolde faeces is één van de uitdagingen van copromicroscopie bij kleine herkauwers om GIN te detecteren op de boerderij zelf met draagbare veldkits.