UNIVERSITÀ DEGLI STUDI DI NAPOLI
"FEDERICO II"

PhD Thesis

A FLOTAC and Mini-FLOTAC automated system for parasitological diagnosis in animals. New tools for Faecal Egg Count of gastrointestinal nematodes in ruminants

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List of abbreviations

ABZ	Albendazole
AEC	Auto Exposure Control
AGC	Auto Gain Control
AI	Artificial Intelligence
AR	Anthelmintic resistance
AWB	Auto White Balance
BZ	Benzimidazoles
CCC	Lin's concordance correlation coefficient
CI	Confidence interval
CPG	Cysts per gram of faeces
CV	Coefficient of Variation
COMBAR	COMBatting Anthelmintic Resistance in Ruminants
D0	Day 0 (pre-treatment)
D14	Day 14 (post-treatment)
DISCONTOOLS	Disease Control Tools
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram of faeces
EU	European Union
FBZ	Fenbendazole
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
FS	Flotation solution
FS2	Satured sodium chloride
GIN	Gastrointestinal nematodes
GI strongyles	Gastrointestinal strongyles
IVM	Ivermectin
KFM	Kubic FLOTAC Microscope
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	fourth-stage larvae
LiHRA	Livestock Helminth Research Alliance
LPG	Larvae per gram of faeces
McM	McMaster
ML	Machine Learning

List of abbreviations

OPG	Oocysts per gram of faeces
PCB	Printed Circuit Board
PE	Parasitic Elements
PGE	Parasitic gastroenteritis
qPCR	Quantitative Polymerase Chain Reaction
RPi	Raspberry Pi
SD	Standard deviation
SG	Specific gravity
SOP	Standard operating procedures

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CHAPTER III

Abstract

Up to date, the copromicroscopic techniques are the reference procedures for the diagnosis of helminths and protozoa in animals and humans. It's fundamental to use a sensitive, accurate, precise, standardized and rapid diagnostic technique for the control and prevention of parasitosis, as well as to detect resistance phenomena.
Since the 1990s, the Unit of Parasitology and Parasitic Diseases of the Department of Veterinary Medicine and Animal Production of the University of Naples Federico II has activated an important line of research, finalized to develop highly sensitive, accurate and precise diagnostic techniques, safe for operator. Therefore, the FLOTAC techniques were conceived, developed (patented in 1999) and validated through different comparisons with the most commonly methods used in veterinary field (e.g. direct smear, Cornell-Wisconsin, McMaster) and in human field (e.g. Ridley and Kato-Katz). The FLOTAC techniques are highly sensitive, accurate and precise, however, require equipped laboratories to be performed that are not always available, especially in endemic countries. Thus, the Mini-FLOTAC techniques, a logical evolution of FLOTAC techniques, were developed. These methods present the same performances of FLOTAC techniques, without require a centrifugation step, resulting more rapid and easier to use. Different studies validated the Mini-FLOTAC techniques that resulted very promising for the diagnosis and counting of parasitic elements in animals and humans, mainly directly in field.
These techniques, as well as all the other Faecal Egg Count (FEC) methods, are affected by the operator's ability (technical skills and working capacity). This PhD project has allowed to implement and automate the FLOTAC and Mini-FLOTAC techniques. A highly innovative digital microscope, the Kubic FLOTAC Microscope (KFM) has been designed and it is currently being validated. This microscope will improve significantly the performances of diagnostic techniques.
In this thesis, technical, mechanical, electronical and informatic aspects of Artificial Intelligence and Machine Learning were described.

CHAPTER I provides an examination of the most common techniques for copromicroscopic diagnosis of gastrointestinal nematodes (GIN) in ruminants. Moreover, the limitations for each technique and the factors that led to the development of the FLOTAC and Mini-FLOTAC techniques are discussed. This chapter highlights also the factors that influence the variability of FEC techniques based on the flotation principle (e.g. method

of preservation of faecal sample, choosing of flotation solution) and that are important for the standardization of methods.

CHAPTER II shows the results of three studies aimed to evaluate and implement the performances of the Mini-FLOTAC techniques.

The first study was aimed to evaluate the sensitivity, accuracy and precision of the Mini-FLOTAC, McMaster and Cornell-Wisconsin techniques for detection of GIN in small ruminants and horses. Eggs extracted from equine and ovine faeces, in known numbers, have been added to equine and ovine negative faeces, in order to obtain four levels of contamination: 10, 50, 200 and 500 eggs per gram (EPG) of faeces. The results showed that the Mini-FLOTAC is the most sensitive, precise and accurate technique at all levels of contamination. McMaster showed 100% sensitivity only at concentrations > 200 EPG, whilst Cornell-Wisconsin significantly underestimated EPG at all levels of contamination. However, a different percentage of egg recovery was detected between ovine and equine faeces, in fact, when eggs of GINs isolated from positive ovine faeces were add to negative ovine faeces, the recovery percentage was 100%, whilst when, eggs isolated from ovine faeces were added to equine faeces, the recovery rate was 91.0%. This difference could be due to the different consistency of the faeces of the two hosts; the horse's faeces, in fact, have a high presence of fibers which could affect the recovery of the eggs.

The second study was aimed to validate the pool strategy for the analysis of cattle faeces in laboratory and on farm in Italy and France. In addition, to carry out the FEC/ Faecal Egg Count Reduction (FECR) of the GIN, directly on farm, a portable kit was used, that consists of the Fill-FLOTAC, the Mini-FLOTAC, floating solution and a portable microscope. Four pools were prepared with a different number of individual samples (5, 10, total in the laboratory and in the company). The results of this study showed that the pooling strategy and the use of a portable FEC kit can be considered quick and inexpensive procedures that can be used for FEC/FECRT both in the laboratory and on farm.

In the third study, the specificity, accuracy and precision of the Mini-FLOTAC and McMaster techniques (at two different reading levels, grids and chambers) for the diagnosis of GIN in cattle were assessed. Negative cattle faeces contaminated with five levels of GIN eggs were used: 10, 50,

100, 200 and 500 UPG. This study was conducted in two laboratories, one in Belgium and one in Italy, in order to evaluate also the reproducibility of the techniques. The Mini-FLOTAC technique was the most sensitive, accurate and precise at all levels of contamination. No significant differences were found between the data obtained in Italy and those obtained in Belgium, thus highlighting the effectiveness of the use of standard operating procedures (SOPs) that allow to obtain reproducible data.

These studies permitted to integrate and strengthen the data on Mini-FLOTAC techniques, in order to develop an automated system for the recognition and counting of GIN eggs in ruminants.

In CHAPTER III is presented the Kubic FLOTAC Microscope (KFM), a digital microscope for the use in field and in laboratory for the reading of FLOTAC and Mini-FLOTAC through the use of Smartphone, Tablet and other newdevices, which allow to send via Internet the captured images for a real-time consultation, in vision of the Tele-Parasitology. The fully automated recognition and counting of the KFM system are in development. In addition, the first validations of the KFM were performed, using faecal samples collected from cattle experimentally infected with *Cooperia oncophora* and *Ostertagia ostertagi* at the Department of Virology, Parasitology and Immunology of the University of Ghent (Belgium).
For each of the thirty samples used, six replicates (for a total of 360 counts) were analyzed with Mini-FLOTAC. The Mini-FLOTACs were read in parallel by the same operator, using a conventional optical microscope and the KFM digital microscope. The results showed that there weren't reading differences with the two different microscopes. Therefore, this new tool combines sensitive, accurate and precise diagnostic techniques, Mini-FLOTAC techniques, with a cutting-edge device for reading samples.

As described in the general discussion (CHAPTER IV), this doctoral thesis has allowed the development of a digital microscope which will be integrated, in the future, in a system that will permit the automation of the FLOTAC and Mini-FLOTAC techniques from the sampling to the reading phase.

Le tecniche copromicroscopiche sono, a tutt'oggi, le procedure di riferimento per la diagnosi di elminti e protozoi sia negli animali che nell'uomo. L'utilizzo di una metodica diagnostica sensibile, accurata, precisa, standardizzata e rapida è di fondamentale importanza, non solo per il controllo e la prevenzione delle parassitosi, ma anche per rilevare fenomeni di resistenza. Dagli anni 90', l'Unità di Parassitologia e Malattie Parassitarie del Dipartimento di Medicina Veterinaria e Produzioni Animali dell' Università degli Studi di Napoli Federico II ha attivato un importante filone di ricerca, finalizzato allo sviluppo di tecniche diagnostiche caratterizzate da elevata sensibilità, accuratezza, precisione e sicurezza per l'operatore. Sono state, quindi, ideate e sviluppate le tecniche FLOTAC (brevettate nel 1999), successivamente validate tramite una serie di comparazioni con le metodiche più comunemente utilizzate in campo veterinario (e.g. Striscio, Cornell-Wisconsin, McMaster) e in campo umano (e.g. Ridley e Kato-Katz). Le tecniche FLOTAC, molto sensibili, accurate e precise, richiedono, tuttavia, l'utilizzo di laboratori attrezzati non sempre disponibili, soprattutto nei paesi in via di sviluppo. Sono state quindi, sviluppate le tecniche Mini-FLOTAC, evoluzione delle tecniche FLOTAC, delle quali conservano le caratteristiche di sensibilità, precisione ed accuratezza, ma non richiedono alcuna fase di centrifugazione, traducendosi in una maggiore semplicità e rapidità. Diversi studi hanno già validato l'utilizzo delle tecniche Mini-FLOTAC che sono risultate molto promettenti per la diagnosi e la conta di elementi parassitari negli animali e nell'uomo, soprattutto in attività di campo.
Queste tecniche, come tutte le altre metodiche di Faecal Egg Count (FEC), risentono molto dell'abilità dell'operatore, in termini di competenze tecniche e di capacità lavorativa al microscopio.
Questo progetto di dottorato ha consentito di procedere in questo filone di implementazione ed automazione delle tecniche FLOTAC e Mini-FLOTAC. E' stato quindi progettato, ed è in corso di validazione, un microscopio digitale molto innovativo, il Kubic FLOTAC Microscope (KFM) che consentirà di ottenere un notevole miglioramento delle performances della diagnostica copromicroscopica. In questa tesi se ne descrivono gli aspetti tecnici, di ingegneria meccanica, elettronica, informatica, di Intelligenza Artificiale e di Machine Learning.

Il CAPITOLO I fornisce una disamina delle tecniche più comunemente utilizzate per la diagnosi copromicroscopica nei ruminanti. Inoltre, vengono discussi i limiti di ciascuna tecnica ed i fattori che hanno portato allo

sviluppo delle tecniche FLOTAC e Mini-FLOTAC. In questo capitolo sono stati messi in evidenza anche i fattori che influenzano la variabilità delle tecniche FEC basate sulla flottazione (e.g. metodo di conservazione delle feci, scelta delle soluzioni flottanti), e che sono di importanza fondamentale per la standardizzazione delle metodiche.

Nel CAPITOLO II sono riportati i risultati di tre studi finalizzati a valutare ed implementare le performances delle tecniche Mini-FLOTAC.

Il primo studio è stato finalizzato a valutare l'accuratezza, la precisione e la sensibilità delle tecniche Mini-FLOTAC, McMaster e Cornell-Wisconsin per la diagnosi di nematodi gastrointestinali (NGI) nei piccoli ruminanti e nei cavalli. Uova estratte dalle feci equine e ovine, in numero noto, sono state aggiunte a feci equine e ovine negative, in modo da ottenere quattro livelli di contaminazione: 10, 50, 200 e 500 uova per grammo (UPG) di feci. I risultati hanno messo in evidenza che il Mini-FLOTAC è la tecnica più sensibile, precisa ed accurata a tutti i livelli di contaminazione. Il McMaster ha mostrato una sensibilità del 100% solo a concentrazioni > 200 UPG, mentre la Cornell-Wisconsin ha significativamente sottostimato le UPG a tutti i livelli di contaminazione. Tuttavia, è stata rilevata una diversa percentuale di recupero delle uova; infatti, quando sono state aggiunte uova di NGI isolate da feci ovine positive a feci ovine negative, la percentuale di recupero è stata del 100%, quando, invece, le uova isolate da feci ovine sono state aggiunte alle feci equine, la percentuale di recupero è stata del 91.0%. Questa differenza potrebbe essere dovuta alla diversa consistenza delle feci dei due ospiti; le feci del cavallo, infatti, possiedono un'elevata presenza di fibre che potrebbe quindi influire sul recupero delle uova.

Il secondo studio è stato finalizzato alla validazione della strategia a pool per l'analisi di campioni di feci bovine in laboratorio e in azienda, in Italia e in Francia. Inoltre, per effettuare la FEC/Faecal Egg Count Reduction (FECR) degli NGI direttamente in azienda è stato utilizzato un kit portatile, costituito da Fill-FLOTAC, Mini-FLOTAC, soluzione flottante ed un microscopio portatile. A tale fine sono stati utilizzati pool costituiti con un numero diverso di campioni individuali (5, 10, totale dei campioni in laboratorio e in azienda). I risultati di questo studio hanno dimostrato che la strategia a pool e l'uso di un kit FEC portatile possono essere considerate procedure valide, rapide ed economiche da utilizzare sia in laboratorio che in azienda per la FEC/FECRT.

Nel terzo studio è stata valutata la specificità, l'accuratezza e la precisione delle tecniche Mini-FLOTAC e McMaster (a due diversi livelli di lettura, griglie e camere) per la diagnosi dei NGI nei bovini. Sono state utilizzate feci bovine negative contaminate con cinque livelli di uova di NGI: 10, 50, 100, 200 e 500 UPG. Questo studio è stato condotto in due laboratori, uno in Belgio e uno in Italia, al fine di valutare anche la riproducibilità delle tecniche. La tecnica Mini-FLOTAC è risultata la più sensibile, accurata e precisa a tutti i livelli di contaminazione. Non sono state riscontrate differenze significative tra i dati ottenuti in Italia e quelli ottenuti in Belgio, sottolineando, quindi, l'efficacia dell'utilizzo di procedure operative standardizzate che permettono di ottenere dati riproducibili.
Questi studi hanno permesso di integrare e rafforzare i dati presenti in letteratura relativi alle tecniche Mini-FLOTAC, per poter sviluppare un sistema automatizzato per il riconoscimento e la conta delle uova dei NGI nei ruminanti.

Nel CAPITOLO III viene presentato il Kubic FLOTAC Microscope (KFM), un microscopio digitale da campo e da laboratorio per la lettura del FLOTAC e del Mini-FLOTAC, tramite l'utilizzo di Smartphone, Tablet ed altri dispositivi di ultima generazione, consentendo di inviare le immagini per una consulenza in tempo reale nella vision della tele-parassitologia. Attualmente sono in via di sviluppo il riconoscimento e la conta completamente automatizzati con il KFM. Le prime prove di validazione del KFM sono state effettuate utilizzando campioni di feci raccolti da bovini presso il Dipartimento di Virologia, Parassitologia ed Immunologia dell'Università di Ghent (Belgio) infettati sperimentalmente con *Cooperia oncophora* o *Ostertagia ostertagi*. Dei trenta campioni utilizzati, sono state preparate sei repliche (per un totale di 360) analizzate con il Mini-FLOTAC. Tutti i Mini-FLOTAC sono stati letti in parallelo dallo stesso operatore, utilizzando un microscopio ottico convenzionale e il microscopio digitale KFM. I risultati hanno dimostrato che le letture effettuate con i due diversi microscopi sono sovrapponibili. Pertanto, questo nuovo strumento combina tecniche diagnostiche sensibili, accurate e precise, le tecniche Mini-FLOTAC, con un dispositivo all'avanguardia per la lettura dei campioni.

Come descritto nella discussione generale (CAPITOLO IV), questa tesi di dottorato ha permesso la realizzazione di un microscopio digitale che sarà integrato, in futuro, in un sistema che permetterà di automatizzare le

tecniche FLOTAC e Mini-FLOTAC dal campionamento fino alla fase della lettura.

INTRODUCTION

I. General overview

Up to date, the copromicroscopic techniques are the reference procedures for the diagnosis of helminths and protozoa in animals and humans. It's fundamental to use a sensitive, accurate, precise, standardized and rapid diagnostic technique for the control and prevention of parasitosis, as well as to detect resistance phenomena.
Since the 1990s, the Unit of Parasitology and Parasitic Diseases of the Department of Veterinary Medicine and Animal Production has activated an important line of research, finalized to develop highly sensitive, accurate and precise diagnostic techniques, safe for operator, to give a support to the real needs of territory. Therefore, the FLOTAC techniques were conceived, developed (patented in 1999) and validated through different comparisons with the most commonly methods used in veterinary field (e.g. direct smear, Cornell-Wisconsin, McMaster) and in human field (e.g. Ridley and Kato-Katz). The FLOTAC techniques are highly sensitive, accurate and precise, however, require equipped laboratories to be performed that are not always available, especially in developing countries. Thus, the Mini-FLOTAC techniques, a logical evolution of FLOTAC techniques, were developed. These methods present the same performances of FLOTAC techniques, without require a centrifugation step, resulting more rapid and easier to use. Different studies validated the Mini-FLOTAC techniques that resulted very promising for the diagnosis and counting of parasitic elements in animals and humans, mainly directly in field.
These techniques, as well as all other Faecal Egg Count (FEC) methods, are affected by the operator's ability (technical skills and working capacity).
This PhD project has allowed to implement and automate the FLOTAC and Mini-FLOTAC techniques. A highly innovative digital microscope, the Kubic FLOTAC Microscope (KFM) has been developed for the automated identification and quantification of gastrointestinal nematode (GIN) eggs in ruminants, to avoid bias due to human errors during visual inspection of PE, as well as to reduce time of diagnosis.

In this introduction some information about GINs in livestock and their impact on animal production are reported.

II. Ruminant farming and gastrointestinal nematode (GIN) infection

Large and small ruminant farming has an important economic and socio-cultural role for rural communities around the world (Bettencourt et al., 2015). In the European Union (EU), for instance, there are currently 86.8 million sheep, 12.7 million goats and 88 million cattle (EUROSTAT, 2017). European livestock production is valued at €1,683 billion annually (45% of the total agricultural activity), imploying about 30 milion of people (Animal Task Force, 2019). In particular, ruminant dairying is important to the agricultural economy of the Mediterranean region, which produces 66% of the world's sheep milk (Pandya and Ghodke, 2007), 15.1% of the world's goat milk (FAOSTAT, 2017) and 27% of the world's cow milk (EUROSTAT, 2017). Moreover, an efficient ruminant livestock production is also crucial to meet the increasing demands of meat, especially in developing countries (Asia and Africa) (Animal Task Force, 2019).

Among the factors that negatively affect the livestock production, parasitic infections, in particular due to gastrointestinal nematodes (GIN) continue to influence animal health, welfare and productivity in grazing cattle worldwide (Charlier et al., 2009).

The ranking of GINs 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) and the recent constituitions of the Livestock Helminth Research Alliance (LiHRA; http://www.lihra.eu) and of the STAR-IDAZ International Research Consortium (https://www.star-idaz.net) reinforces the increasing EU's consideration of the impact of these parasites upon animal health, welfare and productivity. Moreover, the negative impact of GIN on livestock farms is further exacerbated by the escalating spread of anthelmintic resistance (AR) (Vercruysse et al., 2018), a phenomenon under the attention of the scientific community and stakeholders as demonstrated by several European initiatives including the COST Action COMBAR (COMBatting Anthelmintic Resistance in Ruminants; https://www.combar-ca.eu/; CA16230), launched to coordinate research on the control of AR in ruminants.

III. Life cycle and epidemiology of GINs in ruminants

GIN, also known as gastrointestinal (GI) strongyles (from the Greek word *strongùlos*: roundish), are a group of helminths, belonging to Nematoda Phylum, that parazite the abomasum, small and large intestines of ruminants. Grazing ruminants are frequently parasitized by multiple species of GIN, which cause the so-called parasitic gastroenteritis (PGE) (Kassai, 1999). The GIN species present in ruminants include *Haemonchus*, *Ostertagia* (*Teladorsagia)*, *Trichostrongylus*, *Cooperia*, *Nematodirus*, *Oesophagostomum*, *Chabertia* and *Bunostomum* (Zajac, 2006). Some key morphological characteristics (length), pre-patent period (days) and location in the host of the genera of GIN that infect 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 ruminants in Europe (from Anderson, 2000; Taylor et al., 2007; Roeber et al., 2013a).

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

In general, with some exceptions (e.g. *Nematodirus*, *Bunostomum*), the life cycle of the GIN genera listed in Table 1 follows a similar pattern as shown in Figure 1 (Roeber et al., 2013a). Sexually dimorphic adults are present in the digestive tract, where fertilized females produce large numbers of eggs which pass in the faeces. Strongylid eggs (70–150 µm) usually hatch within 1–2 days. After hatching, larvae (L1) feed on bacteria and undergo two moults to then develop to ensheathed third-stage larvae (L3s) in the environment (i.e. faeces or grass). The sheath (which represents the cuticular layer shed in the transition from the L2 to L3 stage) protects the L3 stage from environmental conditions but prevents it from feeding. Infection of the host occurs by ingestion of L3s (with the exception of *Nematodirus* for which the infective L3 develops within the egg and of *Bunostomum* for which L3s may penetrate through the skin of the host). During its passage

through the stomach, the L3 stage losses 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/Ostertagia* (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).

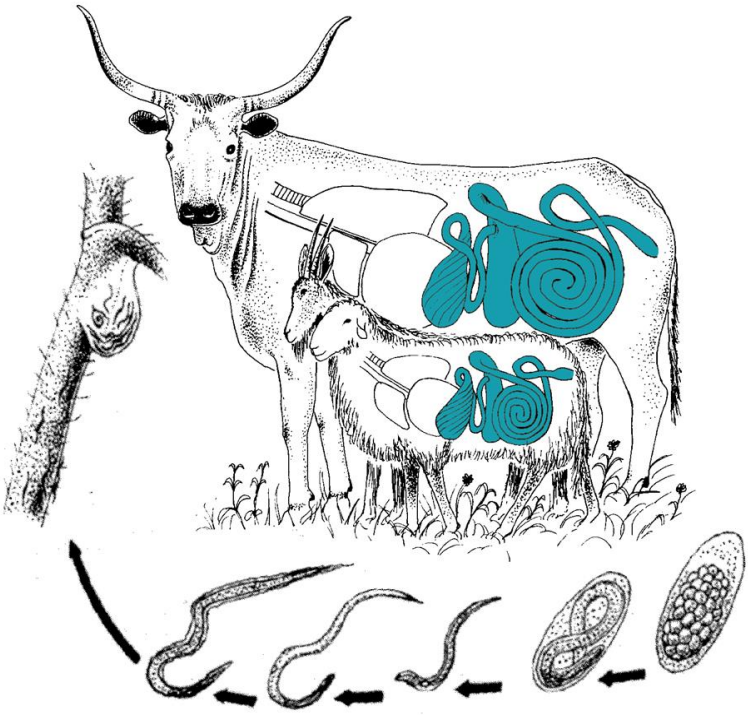

Figure 1 The life-cycle of most genera and species of GINs in ruminants

The large number of prevalence surveys and studies on epidemiology in different 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 ruminants' health and production in warmer, southern areas, while *T. circumcincta/O. ostertagi* is the dominant nematode species of ruminants in temperate and northern regions. *Trichostrongylus* and *Nematodirus* spp. are ubiquitous and their importance varies at local scale. (Morgan and van Dijk, 2012).

IV. Pathogenesis of GINs in ruminants

Different species of GIN can vary considerably in their pathogenicity, geographical distribution, prevalence and susceptibility to anthelmintics (Dobson et al., 1996). *O. ostertagi* and *C. onchopora* located in the abomasum and in the small intestine, respectively, resulted the most pathogenic GINs in ruminants in adult and young cattle across Europe, whilsts in sheep and goats the most pathogenic are *T. circumcincta*, *Trichostrongylus* spp. and *Nematodirus* spp., contributing significantly to PGE (Charlier et al., 2018). *H. contortus* is a highly pathogenic species that can lead to death in heavily infected animals (Besier et al., 2016).
Mixed infections, involving multiple genera and species are common in ruminants and usually have a greater impact on the host (Wimmer et al., 2004). GIN infections cause often chronic infection and associated with hidden subclinical losses such as reduced weight gain, reduced reproductive performances and reduced production (e.g. loss of wool and milk production) (Charlier et al., 2018). Moreover, common symptoms of PGE include , anorexia, diarrhoea, 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; Taylor, 2007; Roeber et al., 2013a). 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 (Fox, 1997; Zajac, 2006: Roeber et al., 2013a). The severity of diseases caused by GIN in ruminants is influenced by several factors such as: i) the parasite species 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; Roeber et al., 2013a). 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).

V. Importance of a reliable diagnosis in ruminants

For most GIN genera/species there is an overlap in size of the eggs; only *Nematodirus* is an exception, because its eggs are sufficiently different for their recognition by size and shape (Figure 2).

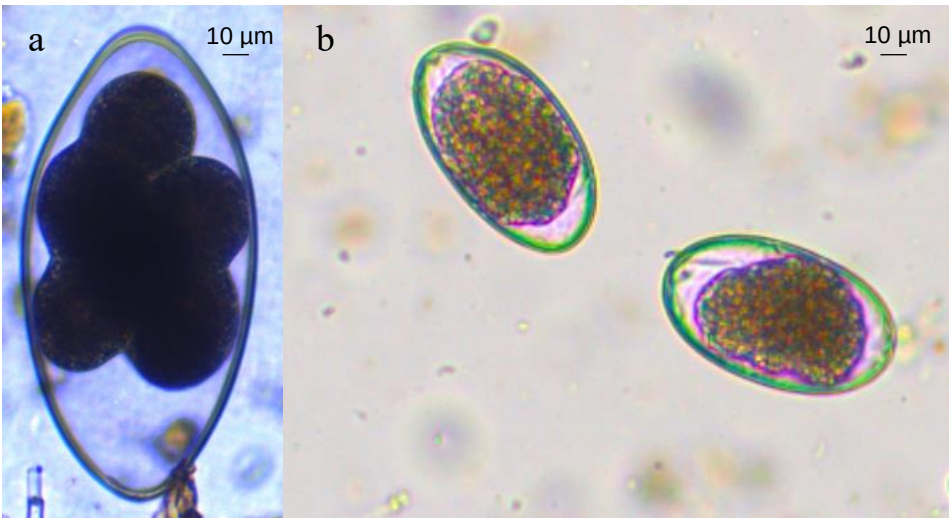

Figure 2 Nematodirus *egg (a) and other GIN eggs (b)*

Therefore, for the identification of different GIN present, eggs recovering has to be followed by faecal culture to identify infective third-stage larvae (L3) of GIN or by molecular techniques (Roeber et al., 2013a).
Altough GIN infections cause important lossess, there are still many gaps in diagnosis. However, an accurate diagnosis of GIN infections is important not only for their control, but also to detect the increasing problem of anthelmintic resistance (AR) (Roeber et al., 2013b). The faecal egg count (FEC) techniques are the commonest laboratory methods for the diagnosis of GIN in ruminants (Roeber et al., 2013b). In literature, numerous methods have been described for the recovering and identifying of GIN eggs, e.g. direct smear, simple flotation in tube, Wisconsin, McMaster, FECPAK (decribed in CHAPTER I). However, there isn't a standardization for these techniques (Kassai, 1999; Roeber et al., 2013b). Moreover, often there is a lack of detailed studies of their diagnostic performances, including the diagnostic sensitivity, specificity, accuracy, precision, reproducibility

and/or repeatability (Roeber et al., 2013a, b). Since, many factors can influence the diagnostic performances of a technique (Cringoli et al., 2010), therefore, it's fundamental to standardize the protocols to obtain reliable results.

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CHAPTER I

The common coprological techniques for diagnosis of gastrointestinal nematode infections in ruminants

1.1 Introduction

The Coproscopy (from the Greek words *κόπρος* = faeces and *-σκοπία* = examen), i.e. the analysis of faecal samples for the presence of parasites (adult or part of them) and/or parasitic elements (PE) (i.e.. eggs, larvae, oocysts and cysts) 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. Copromicroscopic techniques can be either qualitative, providing only the presence/assence of parasites or quantitative, providing also the number of parasitic elements (PEs= eggs, larvae, oocysts and cysts) per gram of faeces (i.e. EPG/LPG/OPG and CPG). After foundation of copromicroscopy by C.J. Davaine in 1857, several copromicroscopic techniques (and devices) have been developed (Fig.1.1), such as the qualitative techniques direct simple flotation in tube (Fulleborn, 1921) and direct centrifugal flotation method (Lane, 1924) orthe quantitative Stoll dilution technique (Stoll, 1923, 1930), McMaster method (Gordon and Whitlock, 1939), Wisconsin flotation (Cox and Todd, 1962) and FLOTAC and Mini-FLOTAC techniques (Cringoli et al., 2010, 2017). Morevover, qualitative and/or quantitative techniques can be performed using an enrichment solution (e.g. sedimentation, flotation) or not (e.g. direct smear), in order to better concentrate the PE and to separate the PE from faecal debris. When tap water is added to faeces, PEs sediment at the bottom of the medium (i.e. sedimentation technique), whilst, when a flotation solution (FS) is added, PEs float at the top of the medium (i.e. flotation technique). Diagnosis of gastrointestinal nematodes (GIN) in ruminants, still relies mainly on qualitative techniques, even if quantitative techniques, also called faecal egg coun (FEC) are very important to determine anthelmintic efficacy/resistance through the faecal egg count reduction test (FECRT) (Vercruysse et al., 2018).

Several variants of these above mentioned techniques, are available in different manuals of diagnostic veterinary parasitology (e.g. MAFF, 1986; Thienpont et al., 1986; Foreyt, 2001; Zajac and Conboy, 2012; Hendrix, 2006). The simplest diagnostic technique was the direct smear, but provided false negative results (Cringoli et al., 2017). One of the first modifications to the faecal smear was the use of sedimentation to concentrate PEs (Rivas, 1928). 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 flotation solution (FS) (Dryden et al., 2005).

The aim of this first Chapter is to provide an overview of the main diagnostic methods for GIN in ruminants, with a focus on FEC techniques. Moreover, limitations and critical gaps of copromicroscopic techniques, as well as the factors affecting their variability, were discussed.

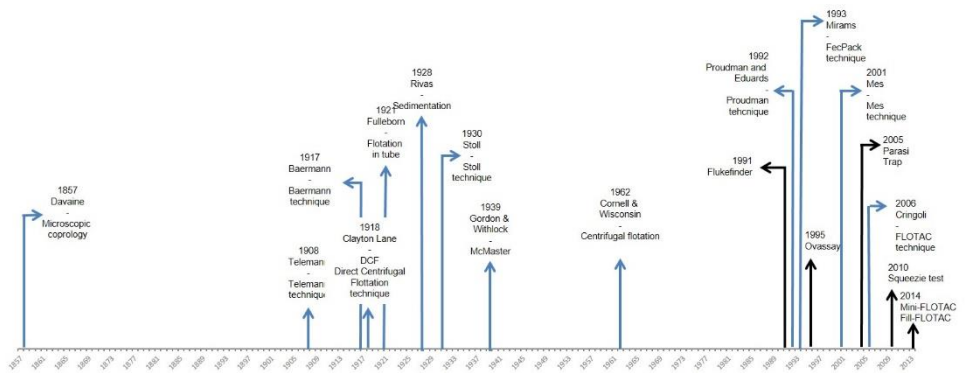

Figure 1.1. Time chart showing the different copromicroscopic techniques (blue line) and commercial devices (black line) developed from 1857 to 2014.

1.2 Flotation techniques and flotation solutions

The methods most frequently used to recover GIN eggs in ruminant faeces are based on flotation.

The PEs float in a FS with a specificity gravity higher (Koutz, 1941; Ballweber et al., 2014). Most of the FSs used in coprology 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 (SG). After preparing any FS, it is mandatory to check the SG with a hydrometer, recognizing that the SG of the saturated solution will vary depending on ambient temperature.

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, 2017). Usually, in the manuals of diagnostic parasitology 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, 2017). Instead, interactions between the elements within a floating faecal 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.

As a rule of thumb, it is noteworthy that:

- Different FSs with the same s.g. do not produce the same results with respect to the same PE, even when the same technique is used.
- A given FS, which might be highly efficient with respect to a given PE, using a given technique, does not produce the same results if the technique is changed.
- A given FS, which is efficient with respect to a given PE, using a given technique on a fresh faecal sample, does not produce the same results if the method of faecal preservation changes (e.g., frozen, preserved in formalin or SAF, or in other fixatives).
- It may happen that a given FS, which is efficient with respect to a given PE, using a given technique, does not produce the same results if the diet of the host changes.

It follows that when a copromicroscopic technique based on flotation is employed, each PE must be considered independently with respect to the FS, the technique and the method of faecal preservation used. What is known for a given PE cannot be readily translated to a 'similar' PE, or to the same PE when the technique or the faecal preservation method changes.

Therefore, especially 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.

Rinaldi et al. (2011) performed a calibration for GIN detection in sheep and suggested that the best FS is sodium chloride (NaCl) with a low specific gravity (1.200) (Table 1.1).

Table 1.1. Flotation solution and recipe used for GIN detection in ruminants.

Flotation solution	Recipe
Sodium chloride (specific gravity 1.200)	1 - Combine 1000 ml of warm water and about 500 grams of salt until no more salt goes into solution and theexcess settles on the bottom of the container. 2 - Dissolve the salt in the water by stirring on a magnetic stirrer. 3 - To ensure that the solution is fully saturated, it should be allowed to stand overnight at room temperature. If the remaining salt crystals dissolve overnight, more can be added to ensure that the solution is saturated. 4 - Check the s.g. with a hydrometer, recognizing that the s.g. of saturated solution will vary slightly with environmental temperature

1.2.1 Flotation in tube

In literature, many diagnostic techniques, using different FSs were developed (Fulleborn, 1921; Stoll, 1923, 1930; Gordon and Whitlock, 1939; Whitlock, 1941; Eigenfeld and Schlesinger, 1944; Seghetti, 1950; Mayhew, 1962; Slocombe, 1973; Rossanigo and Gruner, 1991; Presland et al., 2005; Cringoli, 2004; Cringoli et al., 2017). The flotation in tube is the simplest flotation method. The faecal material is mixed with a FS into a tube. Then, a coverslip is placed on the surface of the tube and after 15 minutes the coverslip is removed to examine it under the microscope (MAFF, 1986). The main limit of this technique is that when the coverslip is removed from the top of the faecal suspension tube and then placed on a microscope slide, not all the floated PEs adhere to the underside of the coverslip. For these reasons flotation in centrifuge techniques were developed, e.g. Clayton-Lane, Wisconsin, Cornell-Wisconsin etc.

1.3 Faecal egg count (FEC) techniques

Copromicroscopic diagnosis of GIN in ruminants is usually performed by quantitative (FEC) techniques. All FEC techniques are based on the microscopic examination of an aliquot of faecal suspension from a known volume of faecal sample (Nicholls and Obendorf, 1994). The results are expressed like number of Pes (eggs, larvae, oocysts and cysts) per gram of faeces (i.e., EPG, LPG, OPG and CPG). Below are reported the main FEC

techniques used for GIN detection in ruminants. For each method in Table 1.2 are reported diagnostic and technical performances (e.g. analytic sensitivity, accuracy and precision in assessing FECs, timing and ease of use), strengths and limitations (Cringoli et al., 2017).

Table 1.2 Characteristics (diagnostic and technical performance) and main limitations of different copromicroscopic techniques used for the diagnosis of helminth infections in ruminants

Technique	Diagnostic performance			Technical performance			Main limitation
	Sensitivity	Accuracy	Precision	Cost	Processing time	Equipment needs	
Direct smear	Very low	Very low	Very low	Inexpensive	Fast	Basic laboratory equipment	Gives positive results only if there are high levels of parasites
Simple tube flotation	Very low	Very low	Very low	Inexpensive	Long	Basic laboratory equipment	Only allows for qualitative, not quantitative, diagnosis
Wisconsin	Low	Low	Very low	Inexpensive	Long	Fully equipped laboratories	Lack of precision, owing to the absence of a grid on the coverslip
McMaster	Medium	Low	Low	Expensive	Medium	Basic laboratory equipment	Diagnosis of intestinal protozoa limited to coccidia
FLOTAC	Very high	Very high	Very high	Inexpensive	Long	Fully equipped laboratories	Requires centrifugation steps with two different rotors and so an equipped laboratory
Mini-FLOTAC	High	High	High	Expensive	Medium	Basic laboratory equipment	Detection of some parasites (e.g., trematoda) requires centrifugation and so, for very accurate applications, it is suggested that the FLOTAC technique be used

1.3.1 Stoll technique

The first FEC technique was developed in 1923 by Stoll which published the paper entitled “Investigations on the control of hookworm disease. XV. An effective method of counting hookworm eggs in faeces” (Stoll, 1923). In the Stoll dilution egg-counting technique, a diluent was added to the faecal sample and a known aliquot was withdrawn, so the eggs per gram of faeces (EPG) were determined using an appropriate dilution factor. In 1930 Stoll published a paper “On Methods of Counting Nematode Ova in Sheep Dung” developing the quantitative faecal egg count also in veterinary medicine (Stoll, 1930).

1.3.2 Cornell-Wisconsin technique

The modified Cornell-Wisconsin technique (Egwang and Slocombe, 1981, 1982) is based on flotation in centrifuge and eggs are recovered by means of adding a cover slide to the meniscus of the flotation solution. (Figure 1.2). This method has an analytic sensitivity of 1 EPG. 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, and the absence of a grid on the coverslip (Cringoli et al., 2010; Levecke et al., 2012b).

Figure 1.2. Flotation in centrifuge (Cornell-Wisconsin technique).

1.3.3 McMaster technique

The McMaster technique (Figure 1.3), 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 FSs, sample dilutions and counting procedures, which achieve varying analytic sensitivities (Cringoli

et al., 2004; Roeber et al., 2013a,b). There are at least three variants of the McMaster technique (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). Although this technique is used in many laboratories, however in different studies showed a low sensitivity, accuracy and precision (Cringoli et al., 2017). Moreover, as reported in Cringoli et al., 2004, this tmethod tends to overestimate the GIN, due to the tendency of eggs, during the flotation, to concentrate in the center of the McMaster slide.

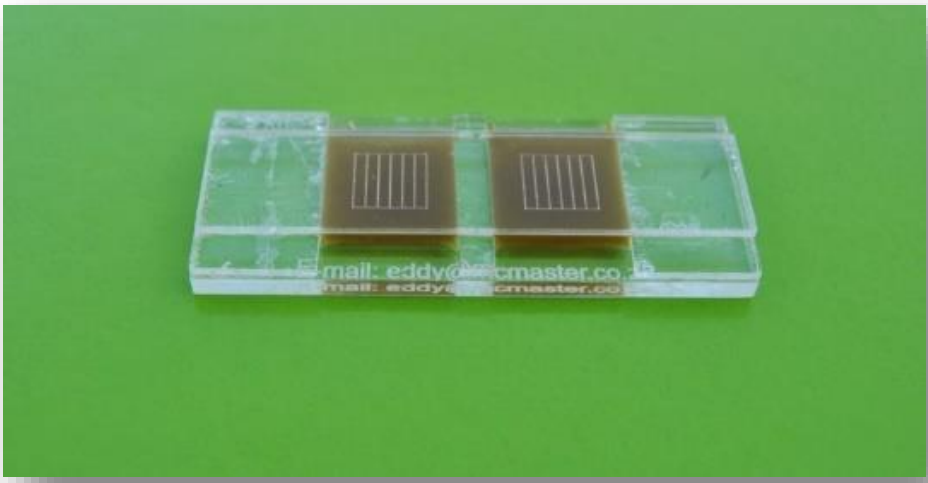

Figure 1.3. McMaster chamber

1.3.4 FECPAK technique

FECPAK (Figure 1.4) was developed in New Zealand to provide a simple "on farm" method for GIN egg counting to make decisions on the need to treat or to determine whether anthelmintics are effective. It is a larger version of the McMaster slide, having a higher analytic sensitivity (usually 10-30 EPG). The apparatus has two flotation chambers and the total volume under the grids is of 1ml. 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). Moreover, this technique is very expensive and it takes a lot of time to be performed.

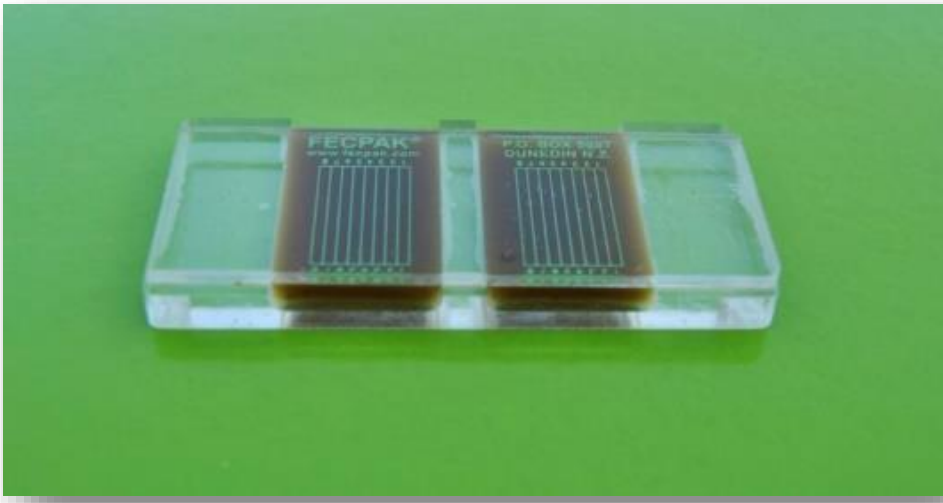

Figure 1.4 FECPAK chamber

1.3.5 FLOTAC and Mini-FLOTAC techniques

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 apparatus is a cylindrical-shaped device made of polycarbonate amorphous thermoplastic. This material has been chosen because of excellent light transmission, high heat resistance, robustness (can be washed and re-used many times) and high-dimensional stability. The FLOTAC apparatus comprises three physical components, namely the base, the translation disc and the reading disc. There are two 5-ml flotation chambers, which are designed for optimal examination of large faecal sample suspensions in each flotation chamber (total volume = 10 ml). There are five accessories, namely the screw, the key, the bottom, the centrifuge adapter and the microscope adapter. These accessories essential for proper functioning of the FLOTAC apparatus during centrifugation and subsequent examination under a microscope.

There are two versions of the FLOTAC apparatus: FLOTAC-100, which permits a maximum magnification of ×100, and FLOTAC-400, which permits a maximum magnification of ×400. FLOTAC-400 is a further development and improvement over FLOTAC-100, as it allows microscopic diagnosis at a four fold higher magnification compared with FLOTAC-100, which is necessary for the detection of intestinal protozoa. FLOTAC-100, however, is still recommended for the diagnosis of helminth eggs and larvae, and for teaching purposes, because the reading disc is considerably thicker and hence more robust than the one used in FLOTAC-400, and because the flotation chambers can be filled more easily.

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 basic steps of the FLOTAC technique are showed in Figure 1.5.

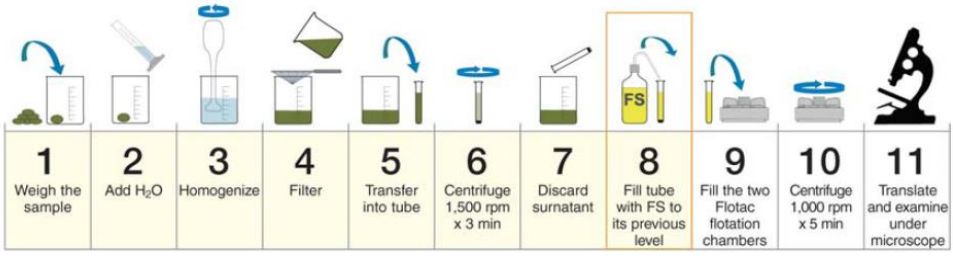

Figure 1.5 The steps of the FLOTAC technique

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 FSs 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 centrifugation steps of the sample with a specific device, equipment that is often not available in all laboratories. 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, that is a logical evolution of the FLOTAC techniques having an analytic sensitivity of 5 EPG (Cringoli et al., 2017). It is a easy-to-use and low cost method, which does not require any expensive equipment (i.e. centrifugation requirement) or energy source, so to be comfortably used to perform FECs (Cringoli et al., 2017) allowing easy transfer and very simple application. The Mini-FLOTAC apparatus is a disk-shaped device made of polycarbonate amorphous thermoplastic, with an excellent light transmission, heat resistance, robustness, high-dimensional stability, and good electrical insulation properties and it is composed by a base and a reading disk (physical components) and a key and a microscope adaptor (accessories) (Cringoli et al., 2017). There are two 1-ml flotation chambers, which are designed for optimal examination of faecal sample suspensions in each flotation chamber (total volume = 2 ml). The basic steps of the Mini-FLOTAC techniques are showed in the Figure 1.6.

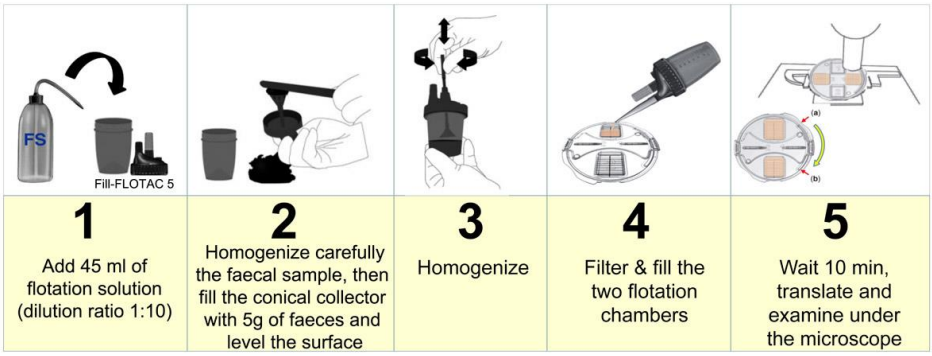

Figure 1.6 The steps of the Mini-FLOTAC technique

The Mini-FLOTAC permits a maximum magnification of 400× and can be very useful also for detection of intestinal protozoa and for the recognition of the details for the speciation of lungworms.

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 (Figure 1.7). FLOTAC, Mini-FLOTAC and Fill-FLOTAC are showed in Figure 1.8. There is a cone on the bottom of the graduated container, which permits homogenization of samples within the closed system of the container. On the top of the lid, there are two holes with screw caps: one central (with a large screw cap) for the collector/homogenizer pole and one lateral (with a small screw cap) for passage of filtered samples. The upper end (handle) of the collector/homogenizer pole is slightly thicker, whereas the lower part is conical and fits over the cone on the bottom of the graduated container. The conical end of the collector/homogenizer pole has a volume of either 2 g (Fill-FLOTAC 2) or 5 g (Fill-FLOTAC 5). As the name suggests, this part of the Fill-FLOTAC allows the collection and homogenization of the faecal sample in the Fill-FLOTAC container before laboratory processing. The filter is in the lower part of the lid. The thin plastic layer is perforated with 250-μm holes to ensure an optimal filtration of the faecal suspension.

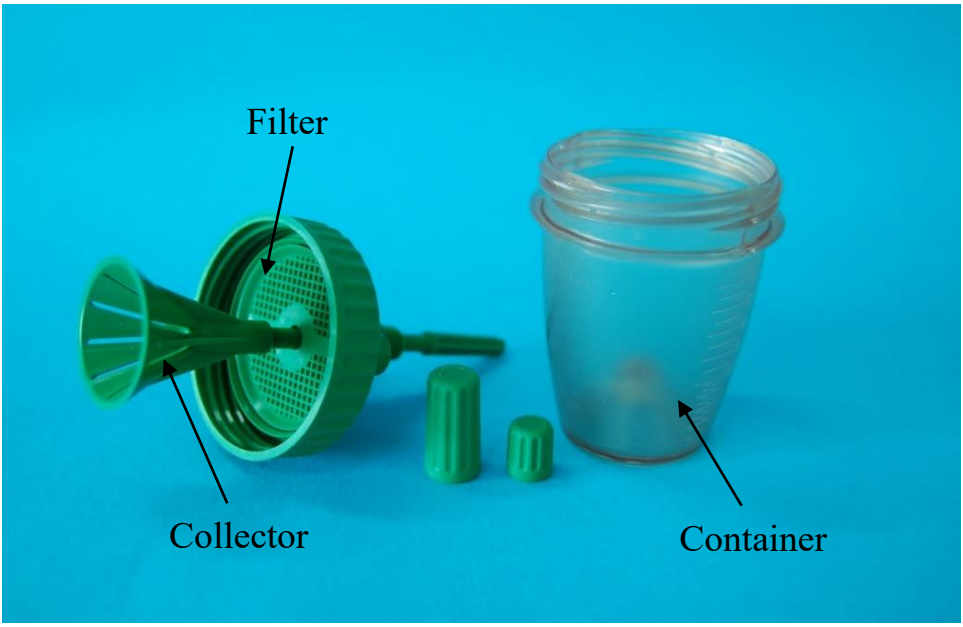

Figure 1.7 The components of the Fill-FLOTAC.

The 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. Morevoer, it is available a commercial kit, called “Mini-FLOTAC portable kit 200 tests” (Figure 1.9) that is very useful for the copromicroscopic diagnosis directly on farm

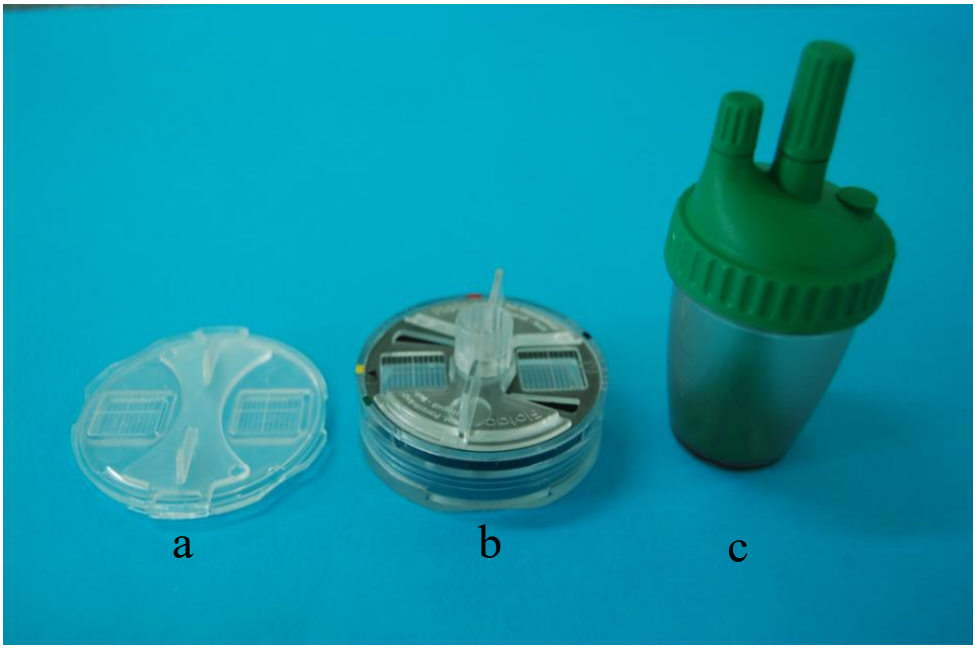

Figure 1.8 (a) Mini-FLOTAC (b) FLOTAC and (c) Fill-FLOTAC apparatus.

Figure 1.9. The components of the "Mini-FLOTAC portable kit 200 tests": (1) Salt for flotation Solution; (2) Tank; (3) Wooden spatula (n = 200); (4) Mini-FLOTAC (n = 4); (5) Fill-FLOTAC (n= 4); (6) Tap; (7) Microscope adaptor (n = 2); (8) Instructions; (9) Devices to disassembly Fill-FLOTAC; (10) Tips for Fill-FLOTAC.

1.4 Conclusions

Although widely used in veterinary parasitology, FEC/FECR techniques are prone to a number of shortcomings.
In fact, there is a clear lack of standardization of FEC techniques and usually each lab uses "its own" method mostly based on the "lab traditions" rather than on the performance (e.g. sensitivity, specificity, reproducibility, negative predictive value), or operational characteristics (e.g. simplicity, ease of use, user acceptability) of the technique (Rinaldi and Cringoli, 2014). Therefore, it is important to standardize the techniques and establish Standard Operating Procedures (SOPs). Based on data of different studies, FLOTAC and Mini-FLOTAC resulted reliable techniques for standardized FEC/FECRT of GIN in ruminants. However, in order to implement and strengthen the data on performances of the Mini-FLOTAC techniques and lay the fundations for the development of an automated system, in

CHAPTER I

CHAPTER II the results of three important studies performed in sheep and cattle are reported.

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CHAPTER II
Improvement of Mini-FLOTAC techniques

1.0 - The recovery of added nematode eggs from the horse and sheep faeces by three methods

Bosco A., Maurelli M.P., Ianniello D., Morgoglione M.E., **Amadesi A**., Coles G.C., Cringoli G., Laura Rinaldi L., 2018. The recovery of added nematode eggs from horse and sheep faeces by three methods. BMC Veterinary Research, 14:7.

1.1 Abstract

Nematode infections in horses are widespread across the world. Increasing levels of anthelmintic resistance, reported worldwide in equine parasites, have led to the creation of programs for the control of nematodes based on faecal egg counts (FEC). To improve nematode egg counting in equine faecal samples and establish whether the matrix of equine faeces or the eggs affect the counts, the analytical sensitivity, accuracy and precision of Mini-FLOTAC (combined with Fill-FLOTAC), McMaster and Cornell-Wisconsin techniques were compared. Known numbers of eggs extracted from equine or ovine faeces were added to egg free ovine and equine faeces to give counts of 10, 50, 200 and 500 eggs per gram (EPG) of faeces. The Cornell-Wisconsin significantly underestimated egg counts and McMaster showed a low analytical sensitivity, revealing 100% of sensitivity only for concentrations greater than 200 EPG. EPG values detected by Mini-FLOTAC did not differ significantly from expected counts at any level of egg density. Mini-FLOTAC combined to Fill-FLOTAC which provides an accurate method of weighing without need for a balance and filtering out debris, could be used for FEC on the farm as well as in the laboratory.

1.2 Introduction

Nematodes which infect horses are clinically important across the world and anthelmintic resistance (AR) is becomingincreasingly prevalent (Andersen et al., 2013). The problem of AR has led to the creation of programs for the control of nematodes based on faecal egg counts (FEC). More accurate and precise FEC methods need to be included in studies evaluating any parasite control program, emphasizing the requirement for simple, reliable and sensitive diagnostic tools and preferably suitable to assess both the intensity of infections and the efficacy of drugs on horse farms (Andersen et al., 2013). Sources of potential error include the method of sampling, flotation solution used, sample dilution, counting procedures (Cringoli et al., 2004; Rinaldi et al., 2011; Levecke et al., 2012), faecal moisture (Roeber et al., 2013), and the storage or preservation of faeces (Rinaldi et al., 2011; Crawley et al., 2016). In order to evaluate which FEC technique is characterized by higher analytical sensitivity (the smallest number of parasitic elements in a sample that can be detected accurately by a given technique), accuracy (how well the observed value agrees with the ‘true’ value) and precision (how well repeated observations agree with one

another), eggs extracted from equine and ovine faecal samples and added to egg free samples were counted by three FEC techniques: Mini-FLOTAC, modified McMaster and Cornell-Wisconsin.

1.3 Material and methods

1.3.1 Faecal sampling

Faecal samples with positive and negative FEC were collected from adult sheep and horses stabled in paddock of farms located in southern Italy. Each sample was analyzed 5 times by the FLOTAC basic technique (Cringoli et al., 2010) with an analytical sensitivity of 1 egg per gram (EPG) of faeces to determine the presence/absence of nematode eggs, i.e. cyathostomes for horses and gastrointestinal nematodes (Trichostrongylus, Haemonchus and Teladorsagia) for sheep. Nematode eggs were extracted from the positive samples using the mass recovery method, i.e., a method that employs 4 sieves of different dimension (1 mm, 250 μm, 212 μm and 38 μm) in order to separate the eggs from the faeces. Then ten aliquots of 0.1 ml each were taken and the number of eggs counted (Godber et al., 2015). A series of cross-contaminations were performed: nematode extracted from horses' faeces were used to contaminate negative horse and sheep faeces and vice versa. The egg suspensions were added to the negative faeces (250 g) and thoroughly homogenized to give four faecal samples (250 g each) for each EPG level (10, 50, 200 and 500).

1.3.2 FECs methods

Each sample was analyzed using satured sodium chloride solution (specific gravity = 1.200) by three FEC techniques: Mini-FLOTAC combined with Fill-FLOTAC (Cringoli et al., 2013; Rinaldi et al., 2014; Whitlock, 1948), modified McMaster technique (Whitlock, 1948) and Cornell-Wisconsin technique (Egwang et al., 1982). After a thorough homogenization from each faecal sample for each EPG level, 60 g were weighted for Mini-FLOTAC, 36 g for McMaster chamber, 36 g for McMaster grid and 60 g for Cornell-Wisconsin. In total twelve replicates were used for each method and for each EPG level (10, 50, 200 and 500) using single faecal samples.
The weight of faeces used, dilution ratio, reading volume and analytical sensitivity of each technique are shown in Table 1.1. Fill-FLOTAC enables the first four step of the Mini-FLOTAC technique i.e. sample collection and

weighing, homogenization, filtration and filling of Mini- FLOTAC chamber (Cringoli et al., 2013; Rinaldi et al., 2014; Cringoli et al., 2017). The repeatability of the 5 g size of Fill-FLOTAC to measure 5 g of faeces using horse and sheep samples was measured 10 times.

1.3.3 Statistical analysis

A coefficient of variation [(standard deviation divided by mean count times) *100] was calculated for each set of replicate counts for each method and level of EPG. The coefficient of variation showed the precision of the method (Kochanowsky et al., 2013) that refers to the closeness of two or more measurements to each other. Mean of eggs (X) showed the accuracy of the method that describe the closeness of a measurement to the true value. The raw counts from each sample were multiplied by the appropriate multiplication factor (5 for Mini-FLOTAC, 50 for McMaster grid, 15 for McMaster chamber and 1 for Cornell-Wisconsin), and then, the mean of the replicate counts for each sample was calculated. The analytical sensitivity of tests across the different levels of egg excretion for each technique was evaluated using line graphs. Boxplots (indicating median, percentiles and outliers) were used to estimate the precision and accuracy of each technique for each of the four levels of egg crosscontamination. A no parametric test, i.e. Spearman rank correlation (rho), was used to examine any association between true and observed egg counts. For each FEC technique at each level of egg count, the percentage recovery was calculated to assess the level of over- or under-estimation of FEC result (measurement error) using the following formula: % egg recovery = 100 - (true FEC - observed FEC) / true FEC * 100. Significance testing was set at $p < 0.05$. Statistical analysis was performed in IBM SPSS Statistics 20.

Table 1.1. Schematic features of Mini-FLOTAC, McMaster (grid and chamber) and Cornell-Wisconsin techniques

FEC Techniques	Amount of faeces used (grams)	Diluition ratio	Reading Volume (ml)	Reading Area (mm2)	Analytic sensitivity (EPG)
Mini-FLOTAC	5	1:10	1.0	648	5
Mc MASTER grid	3	1:15	0.30	200	50
Mc MASTER chamber	3	1:15	1.0	648	15
Cornell-Wisconsin	5	1:3	15	324	1

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

1.4 Results

The study involving 768 counts showed that at all egg concentrations the Mini-FLOTAC and Cornell-Wisconsin had 100% analytical sensitivity (using either sheep or horse faeces contaminated with nematode eggs). Instead, McMaster grid and chamber showed an analytical sensitivity of 100% only for concentrations greater than 200 EPG (the analytical sensitivity ranged from 8.3% to 75.0% at lowest concentration of eggs) (Fig. 1a, b). Spearman's rank correlation showed a significant ($p < 0.05$) positive relationship between observed EPG values and true EPG values for all methods and for all types of crosscontamination, but the Rho values ranged from 0.91 for McMaster grid to 0.97 for Mini-FLOTAC. Additional files show mean of eggs (X), standard deviation (SD) and coefficient of variation (CV%) recovered by Mini-FLOTAC, McMaster and Cornell-Wisconsin for each EPG level and for each contamination. The mean of precision (CV%) and accuracy (X) for each method is presented in Tables 1.2 and 1.3. Figure.1.2 show the boxplot of the observed EPG at each level of egg excretion for Mini-FLOTAC, McMaster grid, McMaster chamber and Cornell-Wisconsin, respectively. The length of boxplots of Mini-FLOTAC technique was very narrow for each contamination level and for all cross-contaminations showing a high precision and accuracy compared to the other techniques.

Sheep faeces had a mean (± standard deviation, SD) of 5.1 ± 0.14 g (maximum 5.1 g, minimum 4.8 g), while horse faeces had an average (± SD) of 5.0 ± 0.11 (maximum 5.2 g, minimum 4.9 g), thus demonstrating a good repeatability of the Fill-FLOTAC for weighing faecal samples. At the lower level of eggs (10 EPG), CV% was high and exceeded 100% in McMaster grid and chamber methods. Furthermore, using McMaster grid and chamber methods were found negative results from the analysis of replicates, whereas the other methods never detected negative results.

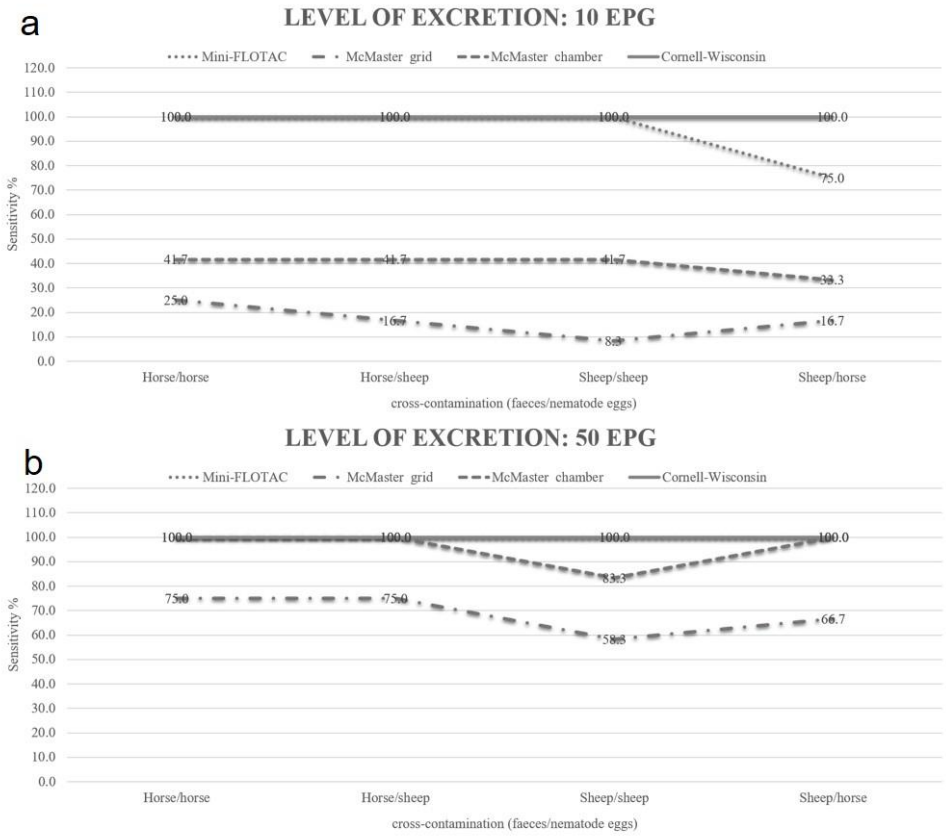

Figure 1.1. Analytical sensitivity (% of positive test results across the replicates) of each FEC technique using nematode egg suspensions of 10 EPG for the four cross-contamination (a) and 50 EPG for the four cross-contamination (b)

Table 1.2 Mean CV% for Mini-FLOTAC, McMaster and Cornell-Wisconsin at the different egg count levels and for each method evaluated in this study

Method	10 EPG	50 EPG	200 EPG	500 EPG
Mini-FLOTAC	49.6%	10.9%	8.1%	3.1%
McMaster grid	248.6%	90.5%	39.9%	17.3%
McMaster chamber	135.6%	51.4%	23.1%	10.9%
Cornell-Wisconsin	33.4%	16.6%	51.8%	5.2%

Table 1.3. Mean number of detected eggs for Mini-FLOTAC, McMaster and Cornell-Wisconsin at the different egg count levels and for each method evaluated in this study

Method	10 EPG	50 EPG	200 EPG	500 EPG
Mini-FLOTAC	9	45	192	409
McMaster grid	8	49	179	492
McMaster chamber	7	39	167	461
Cornell-Wisconsin	4	19	104	248

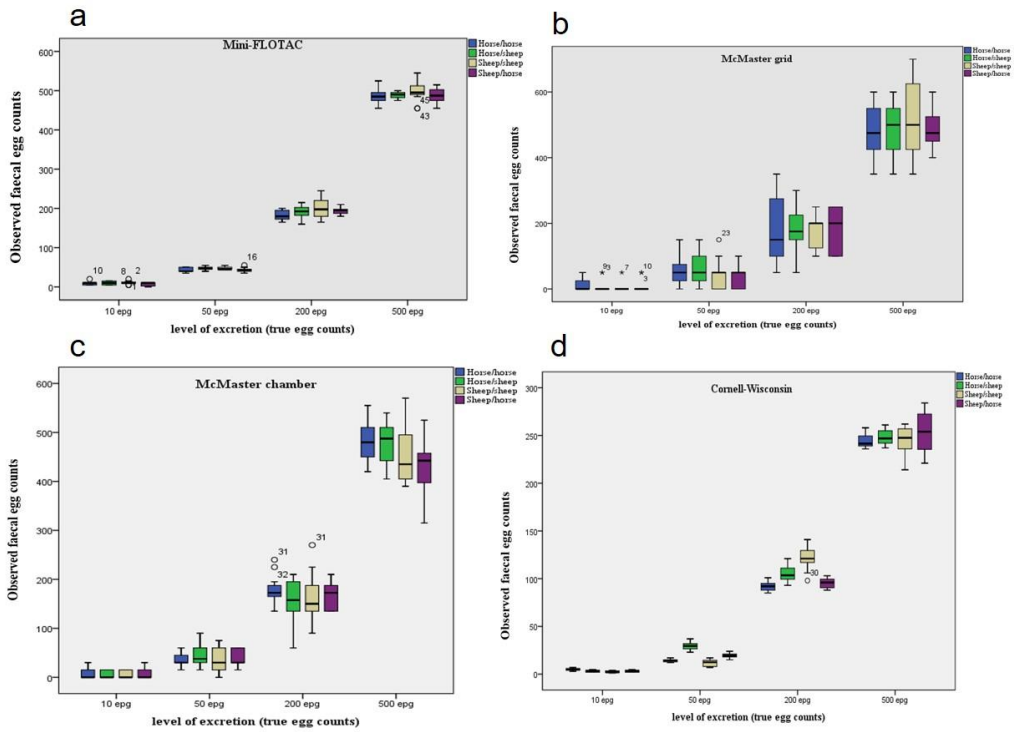

Figure 1.2 Boxplots of observed faecal egg counts (y axis) with: Mini-FLOTAC method (a), McMaster grid (b), McMaster chamber (c), Cornell-Wisconsin (d) for the four levels of egg excretion (x-axis)

1.5. Discussion

Regarding the recovery of eggs, 100% of nematode eggs from sheep were recovered when added to egg-free sheep faeces, but only 91.0% were recovered from horse faeces. There was a significant difference between recovery of nematode eggs of sheep from sheep faeces and from horse faeces. When nematode eggs from horses were added to sheep faeces the recovery was 95.9%, but reduced egg counts (90.5%) were found when added to horse faeces. Noel et al. (2017) performed a study on the percentage of recovery of eggs using Mini-FLOTAC technique for the diagnosis of equine strongyles and recovered 42.6% of the eggs. As discussed by Cringoli et al. (2017), various factors might explain the difference between results presented in this study and results presented by Noel et al. (2017); in fact, one of the main limitations of Mini-FLOTAC technique, as with any

copromicroscopic technique based on flotation (e.g. simple flotation, Wisconsin, and McMaster), is that the selection of fixative and duration of faecal preservation before Mini-FLOTAC analysis, the procedure of egg isolation and the choice of the flotation solution might influence the performance of the Mini-FLOTAC technique, specifically affecting the percentage of parasitic elements recovered (Cringoli et al., 2017). The very poor performance of the Cornell-Wisconsin method indicates that this should not be used in future for counting equine nematode eggs, a conclusion also reached for bovine nematodes (Levecke et et al., 2012). The McMaster technique is adequate if egg counts are greater than 50 EPG, but it is not satisfactory for lower counts which could be important if looking for the beginning AR. These results are similar to Vadlejch et al. (2011) who compared the accuracy and precision of different McMaster methods for diagnosis of *Teladorsagia circumcincta* in sheep and confirmed that this method detected negative samples at lower concentrations. Under-estimation of FEC occurred when the entire McMaster chamber was examined rather than limited to the gridded area (Fig. 2b, c) whereas over-estimation of FEC occurred when the gridded area was examined, due to high multiplication factor. This is in agreement with Cringoli et al. (2004) who observed aggregation of eggs to the center of McMaster slides, Morgan et al. (2005) who described the Poisson distribution of nematode eggs in faecal suspensions and Kochanowsky et al. (2013) that showed that the best limit of detection and analytical sensitivity and the lowest coefficients of variation were obtained with the use of the whole McMaster chamber variant. Only counting eggs in the gridded area appears to account for this aggregation at higher levels of egg densities; the number of eggs present at lower densities, however, was still underestimated. Finally CVs for McMaster grid and chambers were higher than other techniques for ovine and equine faeces, especially for lower counts, as yet reported by Noel et al. (2017). Also Dias de Castro et al. (2017) and Scare et al. (2017) showed that SD lower counts, as yet reported by Noel et al. (2017). Also Dias de Castro et al. (2017) and Scare et al. (2017) showed that SD and CV values for significantly lower for Mini-FLOTAC than McMaster for detection of gastrointestinal nematode eggs in cattle and horses.

1.6. Conclusion

In conclusion, Mini-FLOTAC combined with Fill-FLOTAC which provides an accurate method of weighing without need for a balance and filtering out debris, could be used for FEC on the farm as well as in the laboratory.

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2.0 - Rapid assessment of faecal egg count and faecal egg count reduction through composite sampling in cattle

Rinaldi, L., **Amadesi, A**., Dufourd, E., Bosco, A., Gadanho, M., Lehebel, A., Maurelli, M.P., Chauvin, A., Charlier, J., Cringoli, G., Ravinet, N., Chartier, C. 2019.
Rapid assessment of faecal egg count and faecal egg count reduction through composite sampling in cattle. Parasites&Vectors 12(1), 353.

2.1 Abstract

Faecal egg counts (FEC) and the FEC reduction test (FECRT) for assessing gastrointestinal nematode (GIN) infection and efficacy of anthelmintics are rarely carried out on ruminant farms because of the cost of individual analyses. The use of pooled faecal samples is a promising method to reduce time and costs, but few studies are available for cattle, especially on the evaluation of different pool sizes and FECRT application.
A study was conducted to assess FEC strategies based on pooled faecal samples using different pool sizes and to evaluate the pen-side use of a portable FEC-kit for the assessment of FEC on cattle farms. A total of 19 farms representing 29 groups of cattle were investigated in Italy and France. On each farm, individual faecal samples from heifers were collected before (D0) and two weeks after (D14) anthelmintic treatment with ivermectin or benzimidazoles. FEC were determined individually and as pooled samples using the Mini-FLOTAC technique. Four different pool sizes were used: 5 individual samples, 10 individual samples, global and global on-farm. Correlations and agreements between individual and pooled results were estimated with Spearman's correlation coefficient and Lin's concordance correlation coefficients, respectively.
High correlation and agreement coefficients were found between the mean of individual FEC and the mean of FEC of the different pool sizes when considering all FEC obtained at D0 and D14. However, these parameters were lower for FECR calculation due to a poorer estimate of FEC at D14 from the faecal pools. When using FEC from pooled samples only at D0, higher correlation and agreement coefficients were found between FECR data, the better results being obtained with pools of 5 samples. Interestingly, FEC obtained on pooled samples by the portable FEC-kit onfarm showed high correlation and agreement with FEC obtained on individual samples in the laboratory. This field approach has to be validated on a larger scale to assess its feasibility and reliability.
The present study highlights that the pooling strategy and the use of portable FEC-kits on-farm are rapid and cost-effective procedures for the assessment of GIN egg excretion and can be used cautiously for FECR calculation following the administration of anthelmintics in cattle.

2.2 Introduction

Gastrointestinal nematode (GIN) parasites, also known as gastrointestinal strongyles (Strongylida, Trichostrongyloidea), are amongst the most important productionlimiting pathogens of grazing ruminants in Europe and globally (http://www.disco ntool s.eu) (Charlier et al., 2018). The negative impact of GIN on livestock farms is further exacerbated by the escalating spread of anthelmintic resistance (AR) (Vercruysse et al., 2018), a phenomenon under the attention of the scientific community and stakeholders as demonstrated by several European initiatives including the COST Action COMBAR (COMBatting Anthelmintic Resistance in Ruminants; https ://www.comba r-ca.eu/; CA16230) recently launched to coordinate research on the control of AR in helminth parasites of ruminants. One of the options to make GIN control practices more sustainable is to lower drug application frequency by targeting treatment (TT) to the whole group of animals when infection is high while preserving a pool of unexposed parasites in refugia as free-living stages (Charlier et al., 2014). Diagnosis of gastrointestinal helminth infection is mainly based on the detection of worm eggs through faecal egg counts (FEC) (Charlier et al., 2018). The TT approach requires a relevant method (e.g. FEC) that indicates the worm burden of a given group despite the over-dispersed distribution of parasites within a group of animals (Kenyon et al., 2017). Furthermore, there is an urgent need to obtain better information on the AR status in Europe and FEC are required to estimate anthelmintic efficacy/resistance by the faecal egg count reduction test (FECRT) (Rose et al., 2015). To perform this test, the ideal group size is around 10 to 15 animals (Coles et al., 1992). However, the cost of individual FEC is too high for ruminant farmers and makes veterinarians reluctant to increase FEC-based investigations (Cabaret et al., 2008). As a result, on most ruminant farms, faecal diagnosis is rarely carried out, if at all (Vercruysse et al., 2018). A more regular employment of copromicroscopic monitoring of worm egg excretion could be facilitated by reducing the number of individual FEC analyses through the use of composite (pooled) faecal samples in which equal amounts of faeces from several animals are mixed together and a single FEC is determined from the mixture as a proxy of the group mean FEC. Several studies have been performed in sheep comparing mean individual counts to pooled counts using different pool sizes, ranging from three to ten samples, and different analytic sensitivities of the FEC technique, ranging from 10 to 50 eggs per gram (EPG) of faeces (Baldock et al., 1990; Nicholls et al., 1994; Morgan

et al., 2005; Kenyon et al., 2016; Rinaldi et al., 2014). These studies indicated that pooling ovine faecal samples was a reliable procedure for assessing GIN FEC taking into account the level of FEC, the pool size and the analytical sensitivity of the method (Kenyon et al., 2016). Less is known about faecal pooling in cattle. Ward et al. (1997) in Australia showed a good agreement between mean individual counts (n = 10) and mean composite counts (two pools of five), and George et al. (2017) in the USA successfully tested the single pooling from a group of animals ranging from 9 to 19 individuals (mean number of 15.7). However, these two studies were based either on a composite sample made from two pools of five individual faecal samples or on a single pool of all the individual samples and did not investigate the effect of different pool sizes on the FEC estimation. Besides pooling, field-applicable kits allowing onfarm implementation of FEC with easy-to-use devices to quickly analyze pooled samples are needed by the new generation of veterinarians and farmers to quantify helminth infection, anthelmintic efficacy and AR. Recently, portable FEC kits combined with a mobile phone application have been developed for image capture and specific worm egg quantification in horses and humans (Scare et al., 2017; Slusarewicz et al., 2016; Bogoch et al., 2013). In order to further improve and evaluate the rapid and cost-effective evaluation of FEC and related FECR in cattle, field studies were conducted in order to: (i) further evaluate strategies to assess FEC based on pooled faecal samples (using different pool sizes); and (ii) develop and evaluate a portable FEC-kit in order to perform pooled FEC *on-farm*.

2.3 Material and methods

2.3.1 Study design and sampling

Between June and October 2017, field trials were conducted on a total of 19 cattle farms located in Italy and France. Specifically, in Italy 10 beef cattle farms were included and selected in the Campania and Basilicata regions (southern Italy); cattle were crossbreeds (Limousine, Podolica, Marchigiana). In France, 9 farms were included and selected in Normandy and Brittany regions (north-western France); they were Holstein or Normande breed dairy farms. In both countries, the farms were initially randomly chosen within the selected regions and then the selection was mainly driven by the availability of the farmer and the presence of GIN positive cattle.

Overall on each farm, individual faecal samples (20 g at least) from first or second grazing season heifers (aged from 6 to 20 months) were collected before (D0) and two weeks after (D14) anthelmintic treatment, i.e. ivermectin (IVM, injectable solution, 0.2 mg/kg of body weight) or albendazole/fenbendazole (ABZ/FBZ, oral suspension, 7.5 mg/kg of body weight).

When the number of heifers on a given farm was much higher than 20 and thus exceeded the average value met on most farms, animals were split into similar groups of 10/20 animals and assigned a different treatment.

In Italy, the animals were divided into 2 groups of 10 animals (one group treated with IVM and one with ABZ) on 3 farms; on 3 other farms, 20 cattle were treated with IVM and on 4 farms 20 cattle were treated with ABZ. Similarly, in France, on 2 farms the animals were divided into 2 groups of 18–20 animals, with on each farm one group treated with IVM and the other with FBZ; on 6 other farms, animals were divided into 5 and 6 groups of 11 to 18 animals, respectively; within each farm the groups were assigned to a treatment with either IVM or FBZ. On one farm, a single group of 9 animals was treated with FBZ. Therefore, a total of 29 groups of cattle were available for evaluating the relationship between mean FEC of the individuals and the composite samples, 13 groups (6 treated with IVM and 7 with ABZ) in Italy and 16 groups (7 treated with IVM and 9 with FBZ) in France. The total number of cattle farms, individual faecal samples and pools used for the study are provided in Figure 2.1

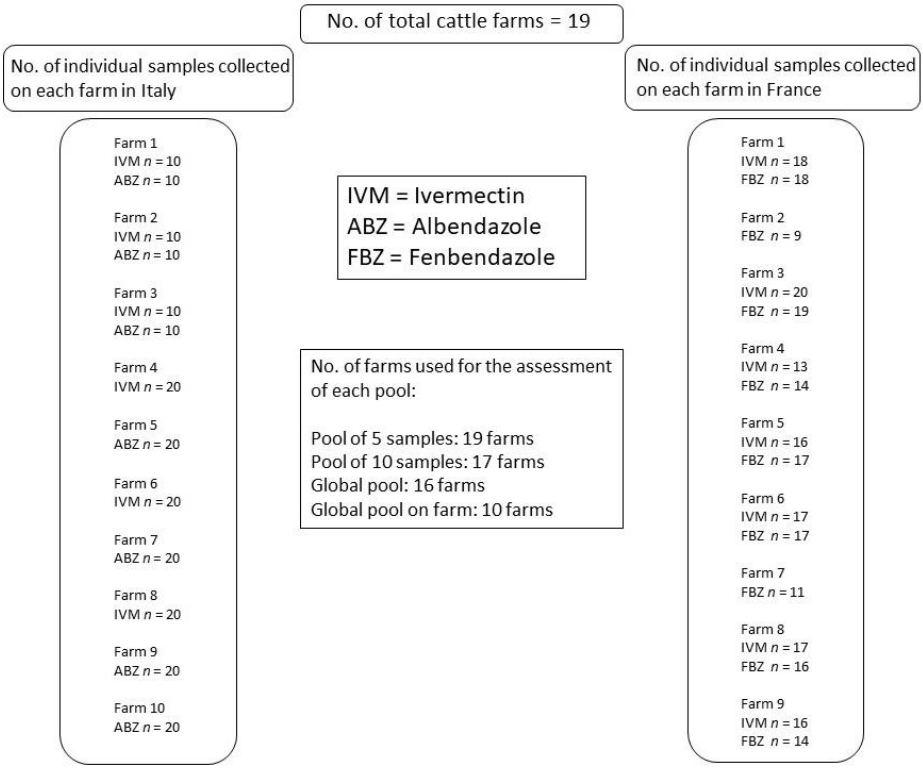

Figure 2.1. The number of Italian and French cattle farms, individual faecal samples and pools used for the study.

2.3.2 Preparation of pooled samples and parasitological analysis

At D0 and D14, bovine faecal samples were analyzed both individually and as pooled samples using the Mini-FLOTAC technique with a detection limit of 5 eggs per gram (EPG) of faeces, using a sodium chloride flotation solution (FS2, specific gravity = 1.200) (Cringoli et al., 2017). Three different pool sizes were used when possible (5 or 10 individual samples, global pooling) according to the protocol described in Rinaldi et al. (2014) and Kenyon et al. (2016). Briefly, each sample was labelled, thoroughly homogenized, individually examined and then composite (pooled) samples were prepared taking approximately 5 g of each sample with the collector of the Fill-FLOTAC (Cringoli et al., 2017).

It should be noted that the predefined pool sizes of 5 and 10 could not always be met at both D0 and D14 due to some practical constraints such as the exact number of animals in the group and an insufficient amount of faeces to perform the analysis of each pools. The actual pool sizes (number of animals from which an individual faecal sample was included) ranged from 3 to 6 for pools of 5 and from 6 to 10 for pools of 10. The global pool was made from all the individuals whatever the group size (ranging from 9 to 20). At D0 and D14, the same animals were sampled and the same pools were prepared. When one sample was missing in a given pool, the corresponding sample was withdrawn before individual FECs were averaged.

2.3.3 FECR on-farm

A portable FEC-kit was developed in order to perform pooled FEC on-farm. The kit consisted of 2 Fill-FLOTAC (for sample collection and weighing, homogenization, filtration and filling) and 2 Mini-FLOTAC devices (Cringoli et al., 2017), the flotation solution (FS2) and a portable (hand-held) microscope with batteries (Celestron, Torrance, CA, USA) for use on-farm. This portable FEC-kit was used on 10 farms to assess a global pool FEC at D0 and/or D14. Briefly, a single pooled sample was prepared taking 5 g of faeces from all individual samples using Fill-FLOTAC and then thoroughly mixed with a spatula in a large beaker. From this pool (90–100 g), a single sample of 5 g was taken by the Fill-FLOTAC and analyzed using the Mini-FLOTAC technique (Cringoli et al., 2017) combined with the reading by a senior researcher under the hand-held microscope.

2.3.4 Coprocultures

For each of the 29 groups of cattle, a pooled faecal culture was performed at D0 and D14, following the protocol described in MAFF (1986). Developed third-stage larvae (L3) were identified using the morphological keys proposed by van Wyk & Mayhew (2013). Identification and percentages of each nematode genera were conducted on 100 L3; if a sample had 100 or less L3 present, all larvae were identified. So, on the total number of larvae identified, it was possible to give the percentage of each genus.

2.3.5 Statistical analysis

The mean FEC of individual and pooled samples were calculated as the arithmetic mean. Correlations between the different measures of FEC were assessed by Spearman's rho correlation coefficient (*rs*), the associated 95% confidence interval (CI) and P-value. Moreover, Lin's concordance correlation coefficients (CCC) and the corresponding 95% CI were calculated to quantify the agreement between the analysis from individual samples and each pool size (including those performed on-farm). Like a correlation, CCC ranges from -1 to 1, with perfect agreement at 1. The strength of agreement was classified as poor, moderate, substantial or almost perfect for CCC values < 0.9, 0.90–0.95, 0.95–0.99 or > 0.99, respectively (McBride GB., 2005).

When examining individual samples, the FECR (%) was calculated according to the formula: FECR (%) = [1 - (arithmetic mean of post treatment individual FECs/ arithmetic mean of pre-treatment individual FECs)] × 100. For each size of pooled samples (5, 10, global), the FECR (%) was calculated as the percent reduction in pooled FEC at D14 compared to corresponding pooled FEC at D0: FECR (%) = [1 - (arithmetic mean of post treatment pooled FECs/ arithmetic mean of pre-treatment pooled FECs)] × 100, the number of pools ranging from 1 to 4. Spearman's rs and Lin's CCC were calculated as above between FECR (%) from individual and pooled samples. In addition, a further correlation analysis (rs and CCC) was done for the calculation of FECR (%) using a "mixed approach", i.e. using FEC on D0 based on pooled samples and FEC on D14 based on individual samples.

The following criterion was used for defining reduced efficacy: FECR < 95% and lower limit of 95% confidence interval < 90% (Coles et al., 1992). The level of significance was set at a P-value < 0.05 for all tests. All statistical analyses were performed using GraphPad Prism v.5 (Graph Pad Software, San Diego, CA, USA) and SPSS Statistics v.23 (IBM, Armonk, NY, USA).

2.4 Results

2.4.1 FEC in individual and composite samples

A total of 200 individual samples were analyzed in Italy and 252 in France. When calculated from individual samples, the mean GIN FEC at D0 and FECR (%) varied between 9.2–359 EPG and 73.3–100%, respectively, providing reasonable variation in FEC and FECR (%) values to be tested in the pooling strategy.

The correlation and the agreement between FEC results from individual means and pool means are reported in Table 2.1 and Fig. 2.2. Overall, the FEC results of pooled samples strongly correlated with those of individual samples regardless of the pool sizes. When focusing on FEC values at D0 or D14, i.e. FEC ranging between 5–400 EPG and 0–69 EPG, respectively, Spearman's rs values were notably lower for D14 FEC values.

The overall level of agreement between the FEC from individual and pool means was substantial for pool of 5 (CCC = 0.99, P < 0.001), pool of 10 (CCC = 0.97, P < 0.001) or global pool (CCC = 0.97, P < 0.001). When considering results separately for D0 or D14, the agreement was substantial for pool of 5 (CCC = 0.98, P < 0.001 and CCC = 0.96, P < 0.001, respectively) and moderate for pool of 10 (CCC = 0.94, P < 0.001 and CCC = 0.95, P < 0.001, respectively) or global pool (CCC = 0.95, P < 0.001 and CCC = 0.94, P < 0.001, respectively).

Regarding the diagnosis directly on-farm including D0 and D14 values, results showed a high correlation (*rs* = 0.94, P < 0.001) and a moderate level of agreement (CCC = 0.93, P < 0.001).

The correlation between FECRs resulting from individual and composite samples showed rs values significant but moderate for pools of 5 samples (*rs* = 0.80, P < 0.001), 10 samples (*rs* = 0.77, P < 0.001) and global pools (*rs* = 0.67, P < 0.001). Similarly, CCC values indicated a poor and decreasing level of agreement for pool of 5 samples (CCC = 0.74; P < 0.001) and global pool (CCC = 0.49, P < 0.001). When considering a mixed determination of FECR using FEC at D0 based on pooled samples and FEC at D14 based on individual samples (Table 2.1), higher values were obtained both for Spearman's correlation coefficients and for CCC values. Specifically, the better results were obtained with pools of 5 samples (*rs* = 1.00, P < 0.001; CCC = 0.97, P < 0.001) and the worst with the global pool (*rs* = 0.80, P < 0.001; CCC = 0.82, P < 0.001). Data were less available for

global pool on-farm and indicated low correlation value (*rs* = 0.68, P < 0.001) and a poor level of agreement (CCC = 0.89, P < 0.001).

Table 2.1 Spearman's rho correlation coefficient (r_s) and Lin's concordance correlation coefficients (CCC) between FEC from individual and pooled samples according the pool size and the FEC values (whole, D0 or D14) and between FECR(%) from individual samples and FECR(%) from individual samples at D14 and pooled samples at D0 according the pool size.

Pool size	N° of pools	r_s	95%CI	CCC	95%CI
Feacal egg count					
Pool 5 samples	58	0.98	0.95-0.99	0.99	0.98-0.99
Pool 10 samples	42	0.97	0.92-0.99	0.97	0.94-0.98
Global pool	58	0.95	0.91-0.97	0.97	0.95-0.98
Global pool *on-farm*	26	0.94	0.82-0.98	0.93	0.88-0.96
Pool 5 samples (D0)	29	0.98	0.93-0.99	0.98	0.96-0.99
Pool 10 samples (D0)	21	0.98	0.90-1.00	0.94	0.86-0.98
Global pool (D0)	29	0.91	0.75-0.97	0.95	0.90-0.98
Pool 5 samples (D14)	29	0.84	0.64-0.94	0.96	0.93-0.97
Pool 10 samples (D14)	21	0.79	0.51-0.92	0.95	0.90-0.98
Global pool (D14)	29	0.69	0.39-0.86	0.94	0.89-0.97
Faecal egg count reduction					
Pool of 5 samples	29	1.00	0.99-1.00	0.97	0.95-0.98
Pool of 10 samples	21	0.88	0.72-0.95	0.82	0.65-0.91
Global pool	29	0.80	0.62-0.91	0.82	0.70-0.90
Global pool *on-farm*	13	0.68	0.20-0.90	0.89	0.85-0.91

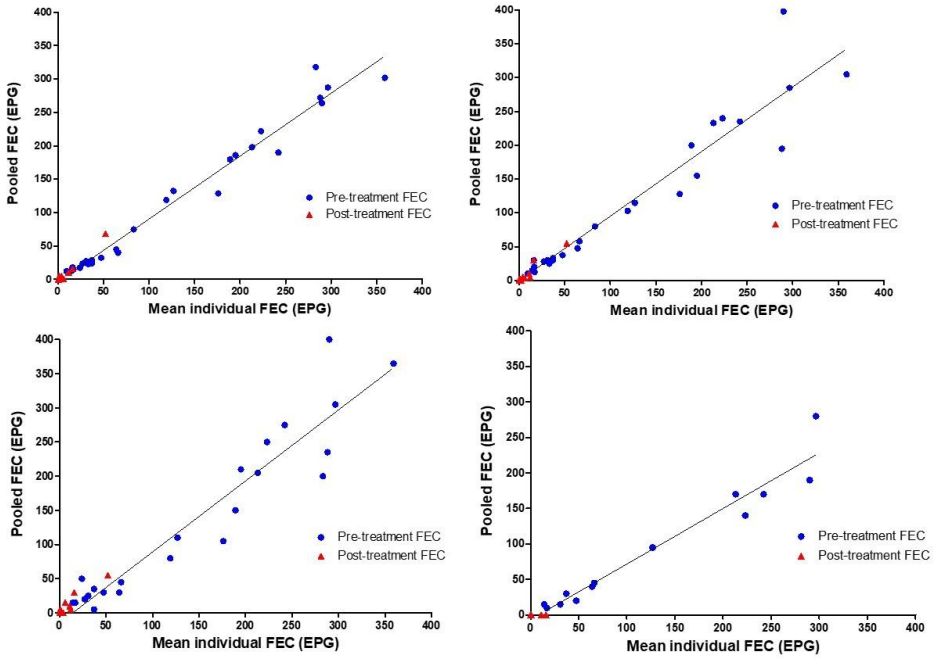

Figure 2.2 The correlation in FEC (pre-treatment and post-treatment) based on the examination of individuals and pools of 5 (a), 10 (b), global pool (c) and global pool analysed directly on-farm (d) in Italy and France

2.4.2 Coprocultures

In Italy, the following GIN genera were detected at D0 (pre-treatment): *Cooperia* (41%), *Trichostrongylus* (20%), *Oesophagostomum* (18%), *Ostertagia* (11%) and *Haemonchus* (10%); at D14 (post-treatment) all samples were negative for GIN larvae. In France, the following GIN genera were detected at D0 (pre-treatment): *Cooperia* (88%) and *Ostertagia* (12%). At D14 (post-treatment), the following GIN genera were detected: *Cooperia* (99%) and *Ostertagia* (1%) on the farms treated with IVM, whilst very few numbers of *Cooperia* and *Ostertagia* were found at D14 on farms treated with FBZ.

2.5 Discussion

Diagnosis of GIN infections by the examination of individual faecal samples, although simple and effective, remains expensive and time-consuming which hampers widespread adoption by farmers. Over the last decade, thanks to the development of new diagnostic approaches and the improvement of the existing ones, considerable progress has been made to improve the performance (e.g. increasing the analytic sensitivity, accuracy and precision) of FEC and FECR in livestock.
However, to increase user-friendliness and uptake of the FEC and FECR by veterinarians and farmers, portable kits are required to make rapid decisions on the need to treat or to determine whether anthelmintics are effective (Charlier et al., 2014).
In addition, promising results have been obtained in pilot studies using pooled faecal samples to decrease the workload and cost of conducting FEC in sheep and cattle (Kenyon et al., 2016; Rinaldi et al., 2014; George et al., 2017). Moreover, in all these studies, as well as in a recent study on a comparison between different FEC methods (McMaster, Wisconsin and Mini-FLOTAC) in four different livestock hosts (cattle, sheep, llamas and horses) (Paras et al., 2018), the good performance of Mini-FLOTAC was emphasized especially when high accuracy is important, such as when measuring FECR.
In the light of these findings, in the present study a practical approach was developed for a rapid and accurate assessment of GIN infection intensity before and after anthelmintic treatment in cattle in Italy and France. The experiment was conducted in parallel in two countries where the susceptibility of GIN could vary as it has been previously mentioned for small ruminants (Rinaldi et al., 2014) but also encompassing potential variation in the laboratory settings where the tests were performed.
The present study provides new insights into standardization of FEC and FECRT on pooled faecal samples by comparing different pool sizes (five samples, ten samples and global) in cattle and the evaluation of a portable kit to perform pen-side FEC.
High correlation and agreement coefficients (Spearman and Lin) were found between the mean of individual FECs and the mean of FECs of three different pool sizes (five samples, ten samples and global) when considering all FEC obtained at D0 and D14. Values were in the same range for the different pools (0.95 to 0.98 for rs and 0.97 to 0.99 for CCC) and indicated that any pooling strategy was efficient. However, when focusing on the

lowest FECs, i.e. those obtained 14 days after anthelmintic treatment; correlations were noticeably lower suggesting a poorer estimation of FEC through pooling, due to a lot of zero data. These poor estimates of FEC at D14 were responsible for a poor FECR calculation.
In contrast, when FEC determination at D14 was based on individual faecal samples, noticeably higher correlation/agreement values were found for FECR, particularly for a pool of five samples. Our results globally confirm the previous data on pooled FEC/FECR obtained in sheep by Kenyon et al. (2016) and Rinaldi et al. (2014) and in cattle by Ward et al. (1997) and George et al. (2017) with different pooling strategies (pools of 5, 10 or 20; global pool of 9–19 animals). In the study of George et al. (2017) involving 14 groups of cattle, the mean individual FEC ranged from 82 to 671 and from 0 to 210 EPG for pre-treatment and post-treatment sampling, respectively whereas the FECR (%) ranged from 53.1 to 100. The authors found very high correlation (rs = 0.92) and agreement (CCC = 0.95) of FECR (%) between individual and global pooling sampling (9–19 animals per pool). Such distributions in mean individual FEC and in FECR (%) have not been found in the context of the French and Italian cattle production. Kenyon et al. (2016) pointed out the importance of the EPG level and the EPG aggregation at D0 for the use of pooled faeces for FECR.
Interestingly, FECs obtained on pooled samples by the portable FEC-kit on-farm showed high correlation and agreement with FECs obtained on individual samples in the laboratory. This field approach has to be validated on a larger scale to assess the feasibility and reliability of FECR calculation *on-farm*.
The present study also confirmed the findings by Geurden et al. (2015) with the full efficacy of ivermectin on cattle farms in Italy and the lack of efficacy on some farms in France.

2.6 Conclusions

The present study highlighted that the pooling strategy and the use of a portable FEC-kit *on-farm* are rapid and cost-effective procedures for the assessment of GIN egg excretion and can be used cautiously for FECR calculation following administration of anthelmintics in cattle. The use of improved FEC and FECR together with harmonization of study design and interpretation (Geroge et al., 2017; Levecke et al., 2018) would allow field surveys to be conducted on a larger scale than today. It would also promote

uptake of diagnostic procedures by veterinary practitioners in order to fill knowledge gaps in the burden of GIN infection and the efficacy of anthelmintics at both the European and global scale. For these reasons, the development of an automated system for reading and counting eggs based on the Mini-FLOTAC technique in the veterinary field is in progress. It uses remote support tools to assist veterinarians and farmers to optimize control strategies so that evidence-based parasite control strategies for livestock can be effectively implemented in the future.

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3.0 - Cattle gastrointestinal nematode egg-spiked samples: high recovery rates using Mini-FLOTAC technique

Amadesi A., Bosco, A., Rinaldi, L., Cringoli, G., Claerebout, E., Maurelli, M.P. 2020. Cattle gastrointestinal nematode egg-spiked samples: high recovery rates using Mini-FLOTAC technique. In press

3.1 Abstract

Faecal egg count (FEC) techniques are commonly used to detect gastrointestinal nematode (GIN) in cattle and determine anthelmintic efficacy/resistance through the faecal egg count reduction test (FECRT). Mini-FLOTAC is one of the techniques recommended for a standardised FEC/FECRT of helminth eggs in cattle. However, only one paper evaluated the recovery rate of GIN eggs by Mini-FLOTAC (compared to McMaster and modified-Wisconsin) in cattle, using only the level of contamination of 200 eggs per gram (EPG) of faeces and using GIN eggs collected from goat faeces to spike faecal samples from cattle. To further study the recovery rate of added GIN eggs from cattle, this study was conducted in two laboratories, one in Belgium and one in Italy, to evaluate sensitivity, accuracy, precision and reproducibility of Mini-FLOTAC and McMaster (at two reading levels: grids and chambers) for the detection of GIN eggs in cattle. In both countries, spiked cattle faecal samples with five different levels of egg contamination (10, 50, 100, 200 and 500 EPG) of GINs were used. The study was performed in both laboratories by the same expert operator and using the same standard operating procedures (SOPs) for Mini-FLOTAC and McMaster. Sensitivity, accuracy and precision were calculated for each technique and for each level of contamination. Moreover, statistical analyses were performed to evaluate differences between techniques. Mini-FLOTAC had a higher sensitivity (100% at all EPG levels for Mini-FLOTAC vs 0-66.6% for McMaster chambers and grids at level <100 EPG) and accuracy (98.1% mean value for Mini-FLOTAC vs 83.2% for McMaster grids and 63.8% for McMaster chambers) and a lower coefficient of variation (10.0% for Mini-FLOTAC vs 47.5% for McMaster grids and 69.4% for McMaster chambers) than McMaster. Moreover, the results of the Mann-Whitney comparison test indicated that there was not a significant difference between the recovery of GIN eggs from the two studies performed in Belgium and in Italy. The high GIN eggs recovery rate detected by Mini-FLOTAC and the similar results obtained in Belgium and in Italy indicated that the diagnostic performance of a FEC technique is strongly influenced by technician dependent variations linked to the accuracy of SOPs regardless of the laboratory environment.

3.2 Introduction

Gastrointestinal nematode (GIN) infections may negatively influence animal health, welfare and productivity in grazing cattle worldwide (Charlier et al., 2009). The negative impact of GIN on livestock farming is further exacerbated by the escalating spread of anthelmintic resistance (AR) in cattle nematodes (Sutherland et al., 2011; Rose et al., 2015; Geurden et al., 2015). In order to limit AR and the misuse/abuse of anthelmintics in cattle, the use of regular diagnostic testing is suggested as one of the options for a sustainable control strategy (Rinaldi et al., 2019). Diagnostic methods for GIN include faecal egg count (FEC) techniques that are commonly used in parasitological research and veterinary practice to indirectly assess GIN burdens and determine anthelmintic efficacy/resistance through the faecal egg count reduction test (FECRT) (Vercruysse et al., 2018). FEC techniques based on easy-to-use devices with high diagnostic performance in terms of sensitivity, accuracy, precision and reproducibility are suggested to perform reliable and exploitable FEC/FECRT in cattle (Paras et al., 2018; Rinaldi et al., 2019).

Mini-FLOTAC is considered a good candidate for a standardized FEC/FECRT of helminth eggs in livestock (Cringoli et al., 2017). This method, in fact, has been compared with different diagnostic techniques, i.e. Cornell-Wisconsin, McMaster and FECPAK, and was shown to be more sensitive, accurate and precise for FEC and FECRT of GINs in sheep (Rinaldi et al., 2014; Godber et al., 2015; Kenyon et al., 2016; Paras et al., 2018; Bosco et al., 2018). Mini-FLOTAC has been also successfully used to perform FEC and FECRT (in lab and on-farm) in cattle (Dias de Castro et al., 2017; George et al., 2017; Paras et al., 2018; Rinaldi et al., 2019). However, only a single study by Paras et al. (2018) evaluated the recovery rate of GIN eggs by Mini-FLOTAC (compared to McMaster and modified-Wisconsin) in cattle. The authors found an accuracy of 70.9%, but eggs used to spike samples were collected from goats and only one level of contamination (i.e. 200 eggs per gram of faeces, EPG) was used. To further investigate the recovery rate of added GIN eggs from cattle, the present paper reports the findings of a study conducted in two laboratories (in Belgium and Italy) to compare Mini-FLOTAC and McMaster (at two reading levels, i.e. grids and chambers) methods, in terms of sensitivity, accuracy, precision and reproducibility, using GIN egg-spiked faecal samples at five different levels of contamination (10, 50, 100, 200 and 500 EPG).

3.3 Material and methods

3.3.1 Study design and sampling

The study was conducted in two laboratories, one in Belgium and one in Italy.
In Belgium, GIN positive and negative faecal samples were collected from Belgian Blue cattle stabled at the experimental farm of the Faculty of Veterinary Medicine (Ghent University). Positive samples were collected from calves (6 months old) experimentally infected with 50,000 L3 of *Ostertagia ostertagi* (n = 2 calves) or *Cooperia oncophora* (n = 2 calves), whilst negative samples were collected from not infected adult (> 24 months) housed cattle.
In Italy, GIN positive and negative faecal samples were collected from Podolian adult cattle (> 24 months) in a commercial farm located in the Salerno province, Campania region. Positive samples were collected from cattle at pasture, naturally infected by different species of GINs, whilst negative samples were collected from stabled cattle. Each sample was analysed in five replicates by the FLOTAC basic technique (Cringoli et al., 2010) with an analytical sensitivity of 1 egg per gram (EPG) of faeces to determine the presence/absence of GIN eggs.
Both in Belgium and in Italy, the positive cattle were used as donor for the extraction of GIN eggs from faeces, using a mass recovery method, i.e. a method that employs 4 sieves of different mesh size (1 mm, 250 μm, 212 μm and 38 μm) in order to separate the eggs from the faeces, as described in Bosco et al. (2018). Eggs were recovered by washing the 38 μm sieve with tap water, and centrifugating the eluate for 3 min at 4000 g. To concentrate the GIN eggs, the supernatant was removed by a water pump and the pellet was resuspended in 5 ml of a 40% sucrose solution. After centrifugation for 3 min at 4000 g, the supernatant was transferred in a new tube, diluted with an equal volume of tap water and centrifuged again for 3 min at 4000 g. The supernatant was removed to reduce the final volume of the egg preparation to 5 ml. Then, ten aliquots of 0.1 ml each were taken, after a through homogenization of egg preparation into two tubes for ten times (avoiding foam formation) for each aliquot to provide a precise counting of eggs (Bosco et al., 2018). Finally, the number of eggs was counted at 100X magnification.
The egg suspensions were added to five confirmed negative faecal samples of 200 g each to obtain five samples with different EPG levels: 10, 50, 100,

200 and 500 EPG. Each sample was analysed, using saturated sodium chloride solution (specific gravity = 1.200), by two FEC techniques: Mini-FLOTAC (Cringoli et al., 2017) and a modified McMaster (MAFF, 1986) technique at two reading levels, i.e. grids and chambers. In total, 12 replicates were used for each method and for each EPG level. From each homogenised faecal sample, for each EPG level, 60 g were weighed for Mini-FLOTAC technique (5 g for each replicate, dilution ratio= 1:10, reading volume = 2 ml, analytical sensitivity = 5 EPG) and 36 g for McMaster technique (3 g for each replicate), reading the two grids (dilution ratio = 1:15, reading volume = 0.30 ml, analytical sensitivity = 50 EPG) and the two chambers (reading volume = 1 ml, analytical sensitivity = 15 EPG). All samples were prepared, analysed and read at 100X magnification by the same expert operator in Belgium and Italy.

3.3.2 Coprocultures

In Italy, faecal cultures were performed in order to identify the nematode genera, following the protocol described in MAFF (1986). Developed third-stage larvae (L3) were identified using the morphological keys proposed by van Wyk & Mayhew (2013). Identification and percentages of each nematode genus were conducted on 100 L3; if a sample had 100 or less L3 present, all larvae were identified.

3.3.3 Statistical analysis

EPG values for each technique and for each GIN infection level were calculated by multiplying the raw counts by the appropriate multiplication factor (e.g. 5 for Mini-FLOTAC, 50 for McMaster grids and 15 for McMaster chambers) and then, the mean of the replicate counts for each sample was calculated. The sensitivity of each method was estimated using the following formula: [(total number of positive samples observed/12, i.e. total number of replicate spiked samples performed for each method and for each level of contamination) *100]. To evaluate the precision of each method, a coefficient of variation (CV) [(standard deviation/mean egg count)*100] was calculated for each set of replicate counts for each method and level of EPGs. Furthermore, the accuracy of each method was determined by the percentage (%) of egg recovery calculated for each level of contamination, using the following formula: % egg recovery = [(observed FEC/ true FEC)*100].

Boxplots (indicating median, percentiles and outliers) were used to show the precision and accuracy of each technique for each of the five levels of egg contamination. Moreover, the Kruskal-Wallis with post-hoc Dunn's test were used to compare the observed/true FEC for each technique and for each level of contamination. Finally, a logistic regression model was performed in order to evaluate the predicted accuracy of each technique. The Mann-Whitney comparison test was used to compare the GIN egg recovery rates by Mini-FLOTAC and McMaster (reproducibility) in the two different laboratories (Belgium and Italy) using different samples from cattle experimentally (Belgium) or naturally infected by different GIN species (Italy), of different ages (calves in Belgium vs adult cattle in Italy) and breed (Belgian Blue vs Podolian). All the statistical analyses were performed in GraphPad Prism v.8 (Graph Pad Software, San Diego, CA, USA). Significance testing was set at $p < 0.05$.

3.4 Results

A total of 360 counts were performed. Mini-FLOTAC technique showed a sensitivity of 100% at all the EPG levels whilst the McMaster technique (reading either grids or chambers) showed a sensitivity of 100% only for levels ≥ 100 EPG. Below 100 EPG the sensitivity of McMaster grids and chambers ranged from 0% to 66.6%. Figure 3.1 and Table 3.1 show the boxplot, the precision (CV %) and the accuracy (%) of the observed mean EPG for each country at each level of egg contamination for Mini-FLOTAC, McMaster grids and chambers. The boxplots of the Mini-FLOTAC technique (Figure 3.1) were very narrow for each contamination level, thus indicating a high precision and accuracy compared to the McMaster grids and chambers.

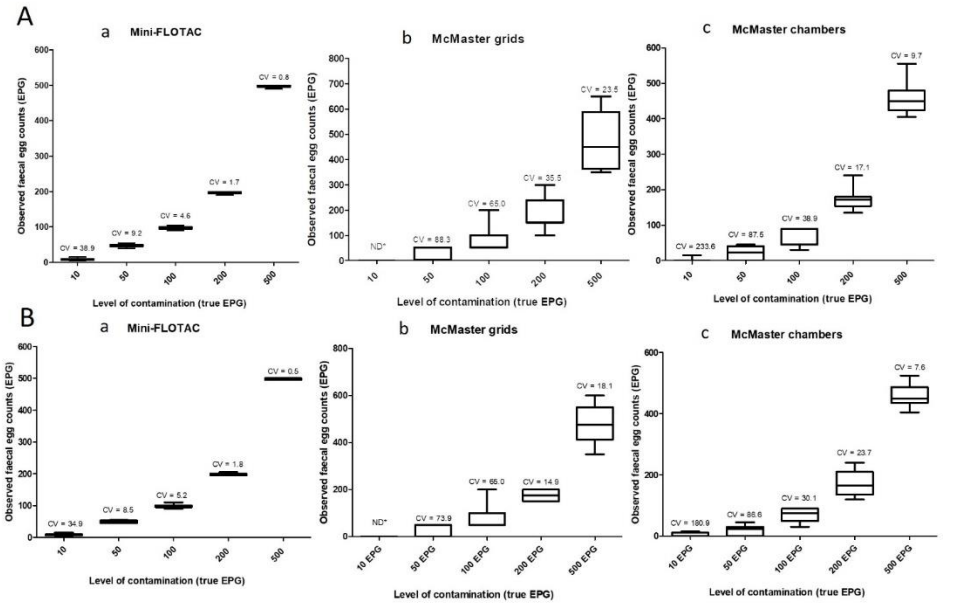

Figure 3.1. Boxplots of observed faecal egg counts (y axis) with Mini-FLOTAC technique (a), McMaster grids (b) and McMaster chambers (c) for the five EPG levels of contamination in Belgium (A) and in Italy (B).

Table 3.1. Mean accuracy (%) of Mini-FLOTAC and McMaster (grids and chambers) at the different EPG levels resulted from the experiment performed in Belgium and in Italy.

FEC method	Accuracy at different EPG level of contamination				
	10 EPG	50 EPG	100 EPG	200 EPG	500 EPG
Belgium					
Mini-FLOTAC	95.8	96.7	97.9	98.5	99.4
McMaster grids	0	58.3	87.5	87.5	95.0
McMaster chambers	25.0	42.5	68.8	88.2	92.0
Italy					
Mini-FLOTAC	95.8	98.3	99.2	99.6	99.5
McMaster grids	0	66.7	87.5	87.5	95.8
McMaster chambers	37.5	40.0	68.8	84.4	91.3

CVs for McMaster grids and chambers were higher than those of Mini-FLOTAC, especially for low counts. The Kruskal-Wallis test showed that there were significant differences for McMaster grids ($P < 0.0001$) and McMaster chambers ($P < 0.0001$) between observed and true EPG values at 10, 50, 100 and 200 EPG levels of contamination, whilst at the level of 500 EPG, only McMaster grids showed no statistically significant difference between observed and true values. This finding was confirmed by the results of the logistic regression (Figure 3.2), the McMaster grids showing a low predicted accuracy related to low FEC, whilst becoming more accurate only when the FEC level increased, i.e. at 500 EPG. Finally, the McMaster chambers had a low accuracy at all the levels of contamination, whilst the Mini-FLOTAC didn't show any significant difference between the observed and the true values at all the EPG contamination levels.

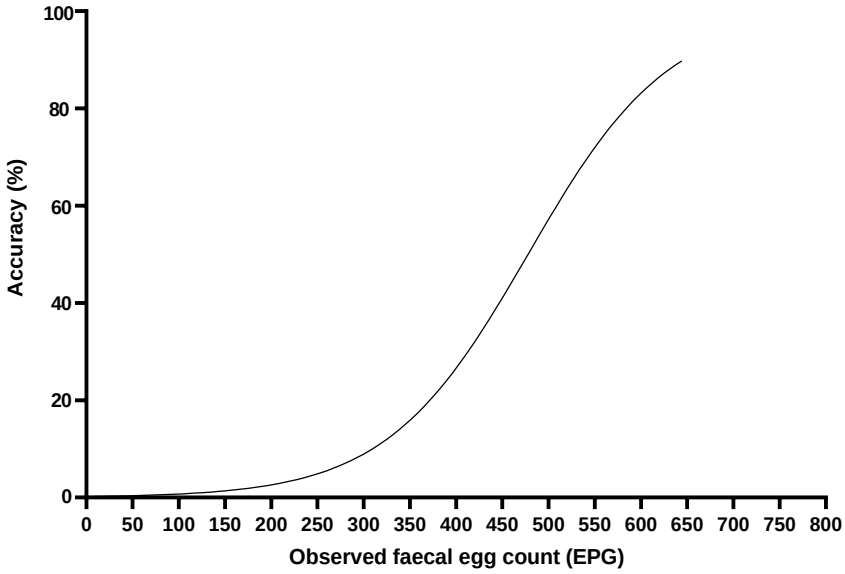

Figure 3.2. The predicted accuracy derived from logistic regression for McMaster technique at grids level.

P values from the Mann-Whitney test ranged from 0.215 to 0.977 (from 10 to 500 EPG levels), showing that there was not a significant difference of the GIN egg recovery rates (either using Mini-FLOTAC or McMaster) obtained in Belgium and in Italy.

3.4.1 Coprocultures

The following GIN genera were detected in the naturally infected samples collected from cattle in Italy: *Cooperia* (52%), *Trichostrongylus* (37%), *Ostertagia* (7%), and *Haemonchus* (4%).

3.5 Discussion

The comparison between Mini-FLOTAC and McMaster for GIN FEC in cattle showed that Mini-FLOTAC had a higher sensitivity and accuracy and a lower CV than the McMaster technique (grids and chambers).
Interestingly, McMaster grids showed higher FECs than McMaster chambers for all levels of contamination (10, 50, 100, 200 and 500 EPG). As described in Cringoli et al. (2004) and Bosco et al. (2018) it may be due to the tendency of eggs, during the flotation, to concentrate in the center of the McMaster slide, with a consequent overestimation of EPGs, especially at low egg counts. Moreover, McMaster showed no statistically significant difference between observed and true EPG only at 500 EPG and at grids level of reading. These results, therefore, showed that the McMaster is not a satisfactory method at low EPG levels, especially when the FECRT is used to evaluate the efficacy of anthelmintics and to detect anthelmintic resistance (Van den Putte et al., 2016; Paras et al., 2018; Bosco et al., 2018).
In this study, the mean percentage of recovery of GIN eggs with Mini-FLOTAC was very high, i.e. 98.1%.
This result is in agreement with Godber et al. (2015) and Bosco et al. (2018) who found a recovering rate of GIN eggs of 100% in sheep spiked faeces. The study by Paras et al. (2018) showed a 70.9% recovery rate of cattle GIN eggs by Mini-FLOTAC that was higher than the values by other techniques (30.9% by modified Wisconsin and 55.0% by McMaster), but lower than the value detected in our study (98.1%). Similarly, Noel et al. (2017) found a 42.6% recovery rate of equine strongyle eggs by Mini-FLOTAC, that was higher that the value from McMaster technique (23.5%). In the study on equine faecal samples by Napravnikova et al. (2019) the accuracy of Mini-FLOTAC was 74.2% (lower than McMaster) for strongyles and 90.3% (higher than McMaster) for ascarids. Finally, Scare et al. (2017) compared an automated FEC using a smartphone with Mini-FLOTAC and McMaster and found a higher accuracy by Mini-FLOTAC (64.5%) compared to McMaster (21.7%) and the smartphone system (32.5%).

As described in Cringoli et al. (2017) and in Norris et al. (2019), the procedure of egg isolation and faeces contamination, as well as the choice of the flotation solution may influence the recovery rates of a technique in any egg-spiking experiment. These factors may have contributed to the high recovery rate of GIN eggs in cattle using Mini-FLOTAC in our study as argued below.

First, as regard the spiking procedure, in our study we spiked cattle faeces with GIN eggs obtained from cattle experimentally (in Belgium) or naturally (in Italy) infected by GINs. This could explain the higher accuracy compared to the findings by Paras et al. (2018) where eggs isolated from goat faeces were used to contaminate cattle faeces. In support of our hypothesis, a recovery rate only of 91.0% was obtained by Bosco et al. (2018) when GIN eggs from sheep were added to horse faeces.

Second, the choice of the flotation solution is very important, because it might influence the performance of the technique and therefore its precision and accuracy (Cringoli et al., 2017). In different studies it has been shown that sodium chloride (specific gravity = 1.200) was the best flotation solution for GIN FEC and it is recommended when using Mini-FLOTAC (Cringoli et al., 2017). Therefore, the low recovery rates found in the above mentioned studies could be due to the inappropriateness of the flotation solutions (i.e. sodium nitrate with a specific gravity=1.25-1.30 (Paras et al., 2018); glucose-NaCl flotation medium with a specific gravity= 1.24-1.28 (Noel et al., 2017; Nàpravnikova et al., 2017; Scare et al., 2017). In our study, CVs of Mini-FLOTAC were lower than the CVs of McMaster grids and chambers for all levels of contamination as reported also in other studies (Godber et al., 2015; Paras et al., 2018; Bosco et al., 2018; Noel et al., 2017; Nàpravnikova et al., 2017; Scare et al., 2017; Dias de Castro et al., 2017; Went et al., 2018). Furthermore, CVs for McMaster chambers were lower than those obtained with McMaster grids, in agreement with Godber et al. (2015) and Bosco et al. (2018). To support these findings, Levecke et al. (2011) and Torgerson et al. (2012) showed that precision increases when analytical sensitivity increases; with the McMaster technique, the variance of EPG estimates between repeated samples of the same faecal sample is inflated, due to the multiplication factor when transforming the raw counts in EPG (Torgerson et al., 2012). Moreover, in this study for all the techniques CVs were lower at higher levels of contaminations, in fact as reported also in Mes et al. (2003) and in Das et al. (2011) the precision increases when the EPG in faecal sample increases.

3.6 Conclusions

Since the sensitivity, precision and accuracy of a FEC depend by many factors, it's very important to establish precise standard operating procedures (SOPs) for FEC techniques, including the flotation solution to use. In fact, it's surprising that diagnostic and research laboratories around the world use different protocols of FEC techniques for their activities. In this view, research priorities should include the development of more scalable, reliable, less labour intensive systems for parasite egg counts for both pen-side and laboratory use (Rinaldi et al., 2019) including methods of automated sample processing and image analysis (Slusarewicz et al., 2016) as indicated in the STAR-IDAZ (https://www.star-idaz.net) diagnostic road map for research on helminths and anthelmintic resistance.

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CHAPTER III

The Kubic FLOTAC Microscope (KFM) and the first validation for the faecal egg count of gastrointestinal nematodes in ruminants

Cringoli, G., **Amadesi, A.**, Maurelli, M.P., Celano, B., Piantadosi, G., Bosco, A., Cesarelli, M., Bifulco, P., Montresor, A., Rinaldi, L. 2020. The kubic flotac microscope (KFM): a new automated system for parasite egg counts. Submitted

3.1 Introduction

Diagnosis has a key role for a correct treatment and an effective control of intestinal helminths and protozoa in humans and animals (Cringoli et al., 2017). Several methods have been described to recovery, identify and count parasitic elements (PEs = eggs, larvae, oocysts, cysts) in faecal samples, e.g. direct smear, simple flotation in tube, Wisconsin, McMaster, formalin-ether concentration, Kato-Katz, FLOTAC, FECPAK, Mini-FLOTAC (Levecke et al., 2011, 2012; Cringoli et al., 2010, 2017). These techniques vary according to their sensitivity, accuracy, precision, reproducibility and repeatability. Moreover, most of these techniques are not standardized, time-consuming, require fully equipped laboratories and are not useful for diagnosis in the field (Cringoli et al., 2017; Sukas et al., 2019).
The use a reliable, low-cost, easy-to-perform, and quantitative diagnostic test is of pivotal importance in order to promote deworming programmes in endemic countries (Cools et al., 2019) and to determine anthelmintic efficacy/resistance through the faecal egg count (FEC) and faecal egg count reduction test (FECRT), in consideration of the growing concern of the emergence of anthelmintic resistance (AR) in both humans (Vlaminck et al., 2019) and animals (Vercruysse et al., 2018; Kaplan, 2020).
In order to improve the diagnostic methods, the Unit of Parasitology and Parasitic Diseases of the Department of Veterinary Medicine and Animal Production developed the FLOTAC and Mini-FLOTAC methods that were compared, since 2007 to day, with the most common copromicroscopic techniques (i.e. direct smear, simple flotation in tube, Cornell-Wisconsin, McMaster and FECPAK) (Figure 3.1) resulting to be a multivalent, quali-quantitative, highly sensitive, accurate and precise alternative method for detection of PEs in veterinary and human field (Cringoli et al., 2010; Cringoli et al., 2017). Moreover, thanks to more than 50 scientific publications on International Journals having a high citation index, FLOTAC and Mini-FLOTAC techniques have been reached a high Technology Readiness Level (TRL).

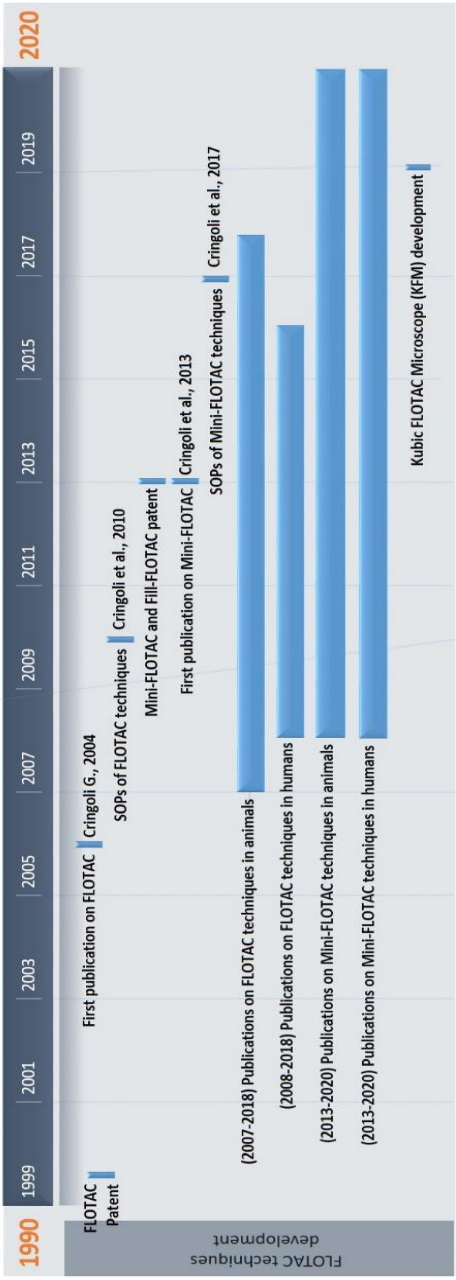


Figure 3.1. Timeline of the evolution of FLOTAC and Mini-FLOTAC.

Standard Operating Procedures (SOPs) of FLOTAC and Mini-FLOTAC techniques in veterinary and human field have been published (Cringoli et al., 2010; Cringoli et al., 2017) and recently, SOPs of Mini-FLOTAC for diagnosis of Soil-Transmitted Helminths (STH) have been included into the WHO Bench Aids for the diagnosis of intestinal parasites (WHO, 2019).
Moreover, to meet needs of new generations of veterinarians and farmers to quickly analyse faecal samples to assess infection intensity and anthelmintic drug efficacy/resistance in ruminants, a pooling sample strategy for livestock was developed and validated using the Mini-FLOTAC techniques either in the laboratory (Rinaldi et al., 2014; Kenyon et al., 2016; George et al., 2017) or directly in the field using the new generation of portable microscopes (Rinaldi et al., 2019). Also, the pooling approach held promise for the rapid assessment of intensity of helminth infections in a programmatic setting in Ethiopia (Leta et al., 2018) but authors suggested further studies to determine when and how pooling can be used.
Even if significant progress has been achieved in diagnosis of intestinal helminths and protozoa in veterinary medicine and public health in recent years, there are further challenges to be addressed, since egg counting results are prone to issues such as operator dependency, method variability and time commitment (Scare et al., 2017; Bosco et al., 2014; Cringoli et al., 2017). In particular, in the present era of technological revolution, automation and artificial intelligence, computerized and automated systems to facilitate and speed up the accurate parasite egg counts are strongly needed and are expected to reduce the time and the number of personnel needed for parasite diagnosis in veterinary and human medicine.

In this Chapter, the Kubic FLOTAC Microscope (KFM), a new computerized automated system for FLOTAC and Mini-FLOTAC for both laboratory and field use, is presented and a first validation for the faecal egg count of gastrointestinal nematodes in ruminants is performed.
Rapidly in the next section some of the semi-automated and automated systems that are developing for parasitological diagnosis are reported, in order to understand the evolution and the development of these new technological devices.

3.1.1 Semi-automated and automated systems for diagnosis in parasitology

Up to date, several studies were reported in literature concerning different smartphone based technologies to magnify objects, to capture images or to perform an automated identification of endo (i.e. protozoa and helminths) and ecto-parasites (i.e. insects and ticks), showing a good potential of application due to their worldwide availability. Saeed and Jabbar (2018) reviewed the applications of various smartphone-based methods and devices developed since 1990 to 2017 for the diagnosis of different parasites in human field (e.g. STH, Schistosoma spp., protozoa, etc.). The authors classified five categories for different smartphone devices as reported in Table 3.1.

Table 3.1 Smartphone-based technologies for parasites detection in humans (principle, hosts, parasites, advantages, limitations, references).

Method	Principle	Hosts	Parasites	Advantages	Limitations	References
Standalone smartphone technology	No any external enhancement such as a lens or a microscope	Humans	*Taenia solium* *Plasmodium falciparum* Ticks	Rapid; low cost; portable	Low sensitivity; only one study for validation	Meena et al., 2013; Scherr et al., 2016, 2017
Lens-mounted smartphone microscopy	The smartphone camera provides a powerful handheld microscope for the identification of parasitic elements	Humans	*Ascaris lumbricoides* Hookworm *Trichuris trichiura* *Schistosoma mansoni*	Rapid; low cost; portable	Low sensitivity; low resolution; validation required	Bogoch et al., 2013, 2014, 2016; Sowerby et al., 2016
Smartphone-assisted manual microscopy	The smartphone is used in conjunction with various microscopic assemblies for the diagnosis of parasites	Humans	*Schistosoma haematobium* *Schistosoma mansoni* *Giardia lamblia* *Plasmodium falciparum* *P. chabaudi*	Rapid; low cost; portable; high specificity	Limited sensitivity; requires a powerful smartphone; validation required	Tseng et al., 2010; Zhu et al., 2011; Ephraim et al., 2015; Coulibaly et al., 2016
Smartphone-assisted automated microscopy	A dedicated smartphone is used in connection with app or algorithm for automated detection of parasites	Humans	*S. haematobium* *G. lamblia* *P. falciparum* *Trypanosoma cruzi* *Loa loa*	Automated; high specificity	Limited sensitivity; validation required	Linder et al., 2013; Zhu et al., 2013; Walker et al., 2014; D'Ambrosio et al., 2015; Koydemir et al., 2015; Slusarewicz et al., 2016
Smartphone-assisted microfluidic technology	A dedicated smartphone is used with the microfluidic lab-on-a-chip devices (LOCDs)	Humans	*P. falciparum* *Anopheles arabiensis* *A. gambiae*	Rapid, portable, high sensitivity	Validation required; DNA amplification required; relatively expensive; expertise required for data analysis	Liu et al., 2012; Stemple et al., 2014

Moreover, several studies were reported in literature concerning the development of semi-automated and automated systems for FEC and the use of new technological devices. Table 3.2 summarizes the principle, the hosts from which faecal samples were collected, the parasites detected, the advantages and limits of each technique.

Table 3.2 Semi-automated and automated systems for parasites detection in humans and animals (principle, the hosts from which faecal samples were collected, the parasites detected, the advantages, limits and references).

Method	Principle	Hosts	Parasites	Advantages	Limitations	References
FECPAK[G2]	High-throughput technological system for *on-field* sample processing	Ruminants; Humans	Gastrointestinal nematodes (GIN); Soil-transmitted Helminths (STH)	Automated detection and count; Remote parasite detection and data on line management	Low sensitivity and accuracy	Mirams, 2016; Rashid et al., 2018; Moser et al., 2018; Vlaminck et al., 2018; Cools et al., 2019; Vlaminck et al., 2019
Parasight system	Based on a fluorescent egg staining and a smartphone to capture images	Horses	Strongyles and *Parascaris equorum*	2.5 minutes test time, less variable and more accurate than McMaster technique	Validated only on horses	Slusarewicz et al., 2016; Scare et al., 2017
Lab-on-disk Platform	Based on a combined gravitational and centrifugal flotation and the use of a converging collection chamber to create a monolayer of eggs	Humans; Pigs	STH, *Schistosoma mansoni; Ascaris suum*	High-quality of images, permitting a good identification and count	High cost; not comfortable to use in field, due to: (i) the need of a minicentrifuge; (ii) the need of electricity; (iii) the big size that doesn't permit to carry easy it	Sukas et al., 2019
Automated robotic system	Based on an automated X-Y stage; the autofocusing and scanning function are based on LabVIEW GUI	Monkey; Dogs; Sheep; Cow	*Trichuris* spp. *Toxocara* spp., strongyle, *Isospora* spp., *Eimeria* spp.	Inexpensive (~US$350), compact, possibility to use fluorescence	The system can be used only with McMaster chamber; not validated	Lu et al., 2018; Li et al., 2019
Automated Diagnosis of Intestinal Parasites (DAPI)	Based on a motorized system to read slides, using a digital camera and a machine learning software	Dogs	*Ancylostoma* spp., *Toxocara* spp., *Trichuris* spp., and *Giardia* spp.	Automated detection of eggs through a machine learning software	High cost; not portable; not validated	Inácio et al., 2020

All the systems reported in Table 3.1 and Table 3.2 showed difficulties in commercialization, mainly due to high costs and/or few published data on validations in field and on lab. For these reasons, there is still the need of the development of a validated and reliable automated system that permits to improve parasitological diagnosis in veterinary medicine and public health.

3.1.2 The Kubic FLOTAC Microscope (KFM)

The Kubic FLOTAC Microscope (KFM) is a portable, automated digital microscope capable of scanning faecal specimens prepared with Mini-FLOTAC or FLOTAC technique (Figure 3.2). The KFM is composed of an electromechanical part that allows a scan of the Mini-FLOTAC or FLOTAC chambers, a software that allow remote interactions and digital image processing supported by Artificial Intelligence (AI) for the recognition of parasite eggs. The mechanical design of the KFM was made with the FREECAD and DESIGN SPARK MECHANICAL tools (Figure 3.3).

Figure 3.2 Kubic FLOTAC Microscope (KFM)

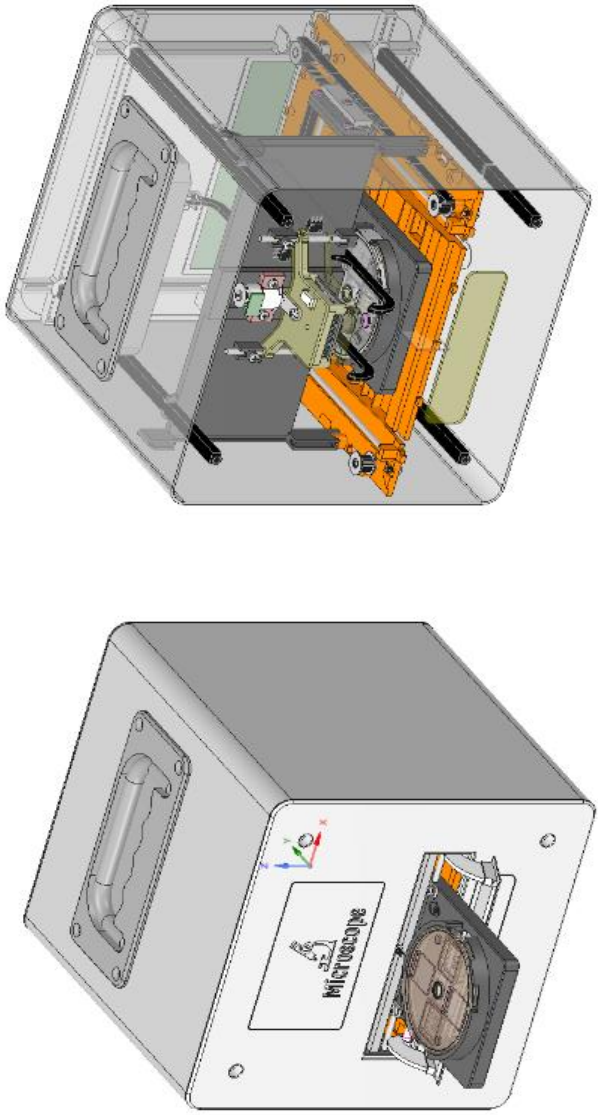


Figure 3.3 FREECAD and DESIGN SPARK MECHANICAL of the KFM

Once opportunely prepared, the FLOTAC or the Mini-FLOTAC device should be inserted in a specific slide-out tray of the KFM (similarly to inserting a DVD into a player). Then, the tray is withdrawn inside and specific 3D landmarks corresponding to the corner of the first flotation chamber of the FLOTAC or the Mini-FLOTAC are automatically located. The KFM scanning device is equivalent to a XYZ motorized stage for microscopy. The 3D positioning system of the motorized stage is based on a simple, non-standard Cartesian motor system solution and is provided of open-loop stepper motors coupled with precision translation stages to achieve accurate 3D motion control (Figure 3.4). A standard, low-cost stepper motor driver based on Arduino nano board and a free Grbl firmware was adopted to control the motors. The motorized stage can automatically move stepwise to entirely scan the two flotation chambers of the FLOTAC or Mini-FLOTAC. For each step a picture (or a Z-stack) is captured by the KFM camera (Figure 3.5).

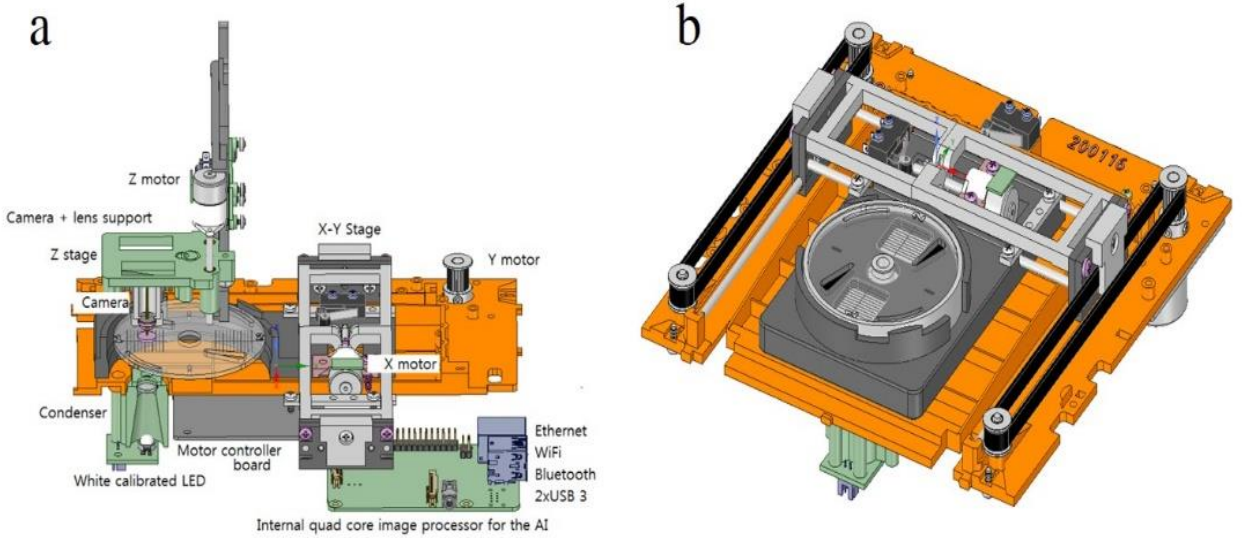


Figure 3.4 a) Schematic diagram showing mechanical, electronical and optical systems of the KFM (b) A particular of the handling motor based on the no-standard cartesian system

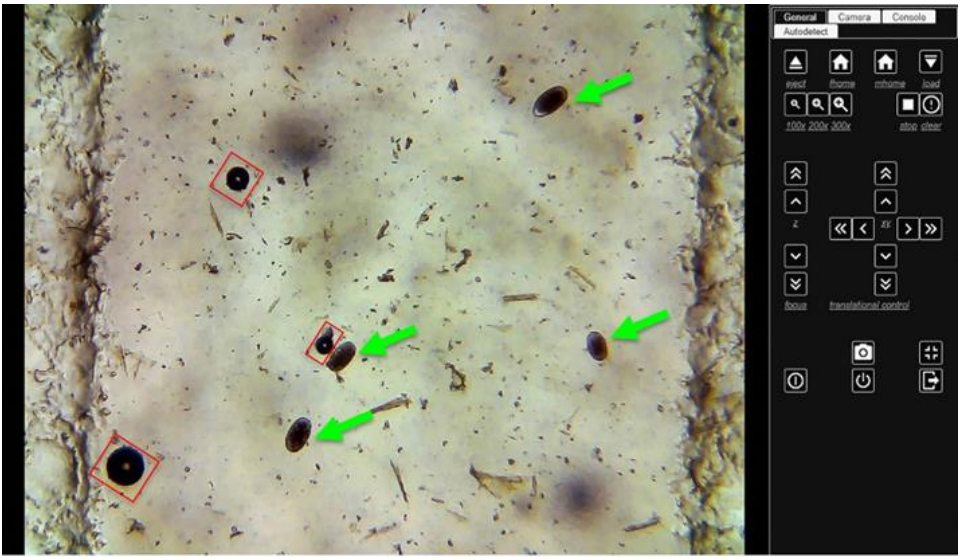
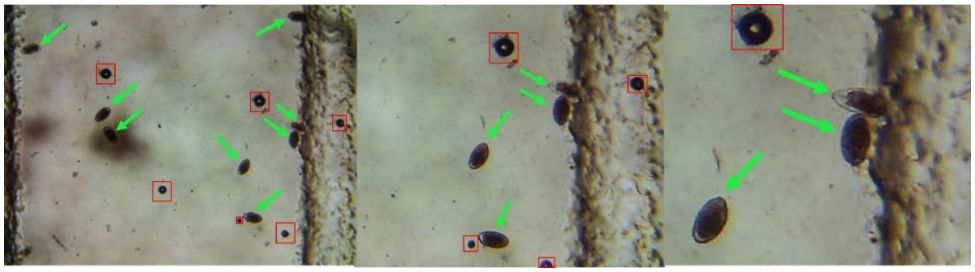


Figure 3.5 An image of a part of a Mini-FLOTAC chamber, captured by technological devices (Smartphone, Tablet or a PC) connected with the microscope that shows GIN eggs (green arrows) and air bubbles (red squares).

The KFM optical part is mainly composed of a LED light source with condenser, which provides brightness adjustment, and a digital camera, which permits adjustable magnification at 100X, 200X, 300X (Figure 3.6). Eventually, the whole surface of the flotation chambers (each 18 mm by 18 mm wide) is photographed. The KFM device has various options for external connectivity: two USB ports that let memorize the captured images (or video); an Ethernet cable connector; a Bluetooth and a Wi-Fi wireless connection, which permits to send the captured images to other computers. In addition, The KFM can be fully controlled via software by an external device, i.e. a Smartphone, a Tablet or a PC. The KFM device can be manually remotely controlled via a web interface; the internal software works on Linux operating system. Such interface can be activated using any web browser. The remote interface supports Android 4.1, iOS 8.0, Windows 7/8/10, MacOS X 10.8. A specific app has been developed to control the KFM directly from mobile phones. The software has two modalities: "Administrator", password protected, for the management of settings and mechanical movements and "Viewer" to allow remote viewing. The KFM device is predisposed to share the collected images in an Internet cloud or to transmit them to expert diagnostic centers for telemedicine diagnosis or consultation (Figure 3.7).



(a) **(b)** **(c)**

Figure 3.6. Digital imaging of GIN eggs (green arrow) and air bubbles (red square) using the KFM with a digital zoom 100X (a) 200X (b) and 300X (c)

Figure 3.7 The Kubic FLOTAC Microscope global connection

3.2 Materials and Methods

3.2.1 Study sampling and analysis

Thirty faecal samples were collected from Belgian Blue and Holstein cattle (6 months old) stabled in a farm of the Department of Veterinary Medicine (Ghent, Belgium) experimentally infected with 50,000 larvae at the third stage (L3) of *Cooperia oncophora* or *Ostertagia ostertagi*.

The Mini-FLOTAC technique was performed using the SOPs described in Cringoli et al. (2017) for ruminants with an analytical sensitivity of 10 eggs per grams of faeces (EPG).
For each sample, six Mini-FLOTACs were performed, using six Fill-FLOTACs filled with a satured sodium chloride flotation solution (specific gravity = 1.200) (Cringoli et al., 2017). To ensure the quality of parasitological examination, Mini-FLOTACs were read with an optical microscope by one operator, then were randomized by a second operator before the reading with KFM to obtain blinding results.

3.2.2 Statistical analysis

A Mann-Whitney test was used to compare the reading with both microscopes. Moreover, the level of agreement was analyzed using the Bland–Altman plots. Finally, the Spearman rank correlation coefficient and a linear regression were calculated between reading with optical microscope and with KFM. All tests were considered statistically significant at $p < 0.05$. All the statistical analyses were performed in GraphPad Prism v.8 (Graph Pad Software, San Diego, CA, USA).

3.3 Results

In total, 180 counts (6 replicates for each sample) using the optical microscope and 180 counts (6 replicates for each sample) using the KFM were performed. Analysis of the samples showed a wide range of GIN eggs (from 1 to 62 eggs), as depicted in Figure 3.8. Based on EPG counting, each sample was assigned to one of three levels of infection: low (< 10), medium (10-25) and high (> 25). The number of counted eggs and mean, for each level of infection and total data are showed in Table 3.3.

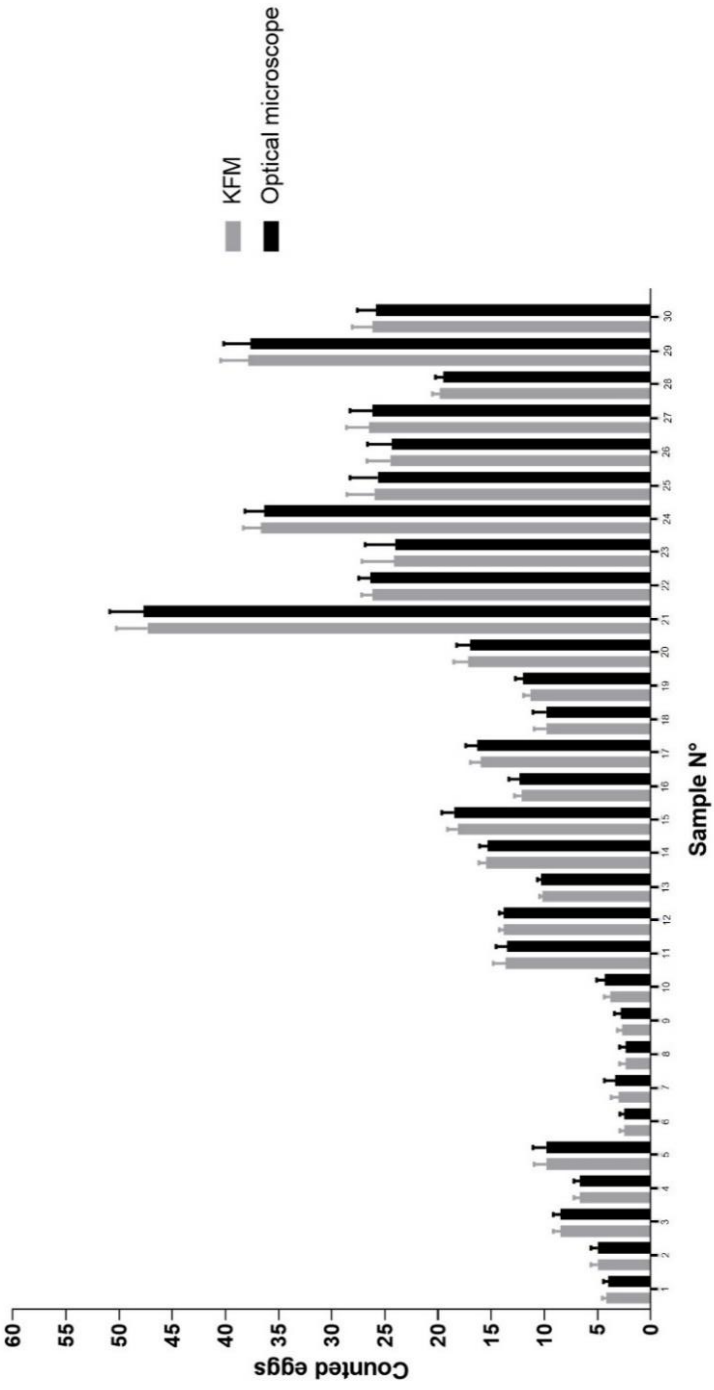


Figure 3.8. Comparison of the reading with the optical microscope and with the KFM .

Table 3.3 Number of counted eggs (sum and mean) for the analysis performed by Mini-FLOTAC using an optical microscope and the KFM at the different egg count levels.

Method	Low*(Sum ± mean)	Medium*(Sum ± mean)	High*(Sum ± mean)	Total data (Sum ± mean)
Optical microscope	296±4.93	834±13.90	1761±29.35	2891±16.06
KFM	291±4.85	827±13.78	1771±29.52	2889±16.05

*Level of infection

The correlation and the agreement between counted eggs results from both reading for each level and total data are reported in Figure 3.9 and Figure 3.10.

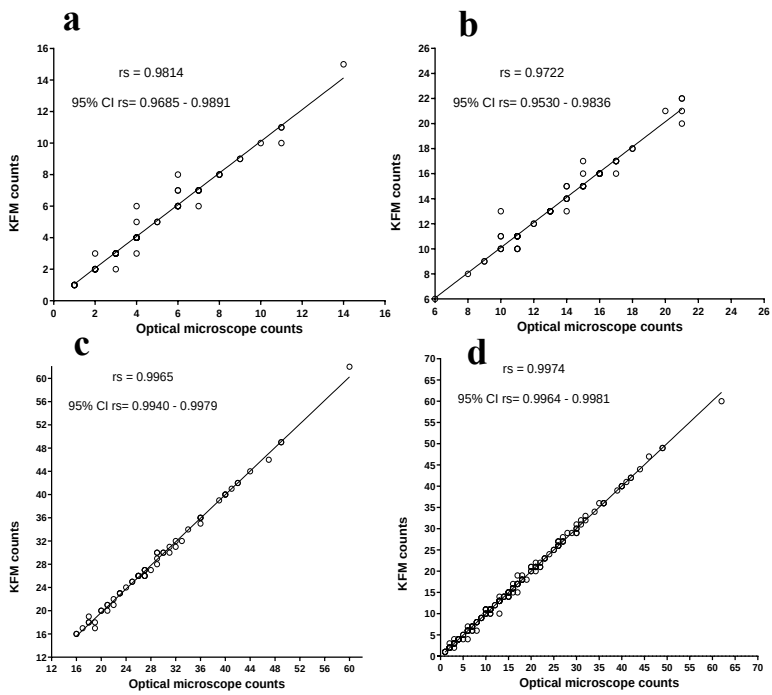


Figure 3.9 The correlation in between number of counted eggs based on the examination of Mini-FLOTAC by optical microscope and KFM for each different level of infection (a) low level of infection (b) medium level of infection (c) high level of infection and for total data (d).

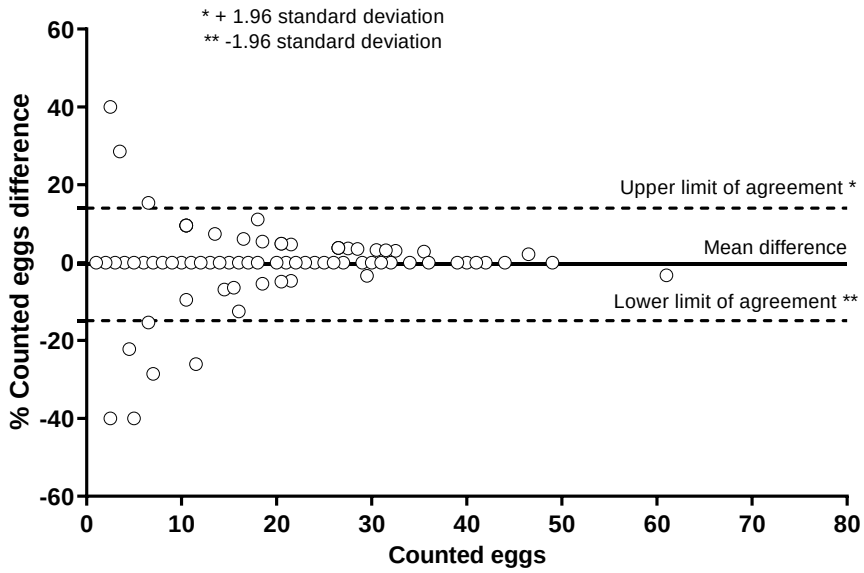


Figure 3.10 Bland-Altman plot of number of counted GIN eggs based on the examination of Mini-FLOTAC by optical microscope and by KFM.

Results showed that there wasn't statistically significant difference between methods of reading, for each level of infection.
Spearman's rho correlation coefficient (rs) analysis revealed a strong correlation between the two readings (rs= 0.9986, p <0.0001) for each sample (Figure 3.9), as well as the Bland-Altman plots (Figure 3.10) that demonstrated an excellent agreement between the two reading approaches (bias= −0.425 ± 7.370 with 95% limits of agreement from −14.8715 to 14.0206).

3.4 Discussion

The KFM presented in this paper is a new system to automate the parasitological diagnosis in high and low-resources settings (Cringoli et al., 2010, 2017) that can be suitable for laboratory routine and field use. The results of the comparison between reading with optical microscope and with KFM showed that there is an almost perfect correlation (*rs* = 0.9974) between the counted GIN eggs. Furthermore, it was demonstrated that the discrepancy between the reading with the KFM and with the optical

microscope is extremely low (−0.425 ± 7.37) and showed that is not affected by the number of eggs counted.

The main advantage of this automated microscope is the combination of sensitive, accurate, precise and standardized diagnostic techniques, FLOTAC and Mini-FLOTAC, with a reliable system to capture and analyse pictures, permitting to reduce human errors and time of reading. Moreover, via the Internet it will be possible to transfer the captured pictures to other laboratories, that could be very useful to create a network of laboratories or to support operators directly in the field as expected by Tele-Medicine and Tele-Parasitology (Scheild et al., 2007; Di Cerbo et al., 2015; Zaffarano et al. (2018).
The KFM can be used: i) without requiring special equipment in laboratories or directly in field; ii) by any operator without a specific training; iii) without requiring high cost.
In addition, the captured images by KFM present a high quality that is fundamental not only for a good recognizing of the PEs, but also for the development of an AI software, described below, that will permit to count automatically the PEs. This next goal will be available in next months, before for GIN in ruminants and later also for other PEs of veterinary and public health importance.
Finally, up to date, the KFM allows to obtain the digital count in 12/15 minutes for each chamber of FLOTAC or Mini-FLOTAC; further studies will be performed to improve the speed of reading and reduce the time for the analysis.

3.5 Future perspectives

3.5.1 Imaging Setup

One of the more important improvement will be the development of a predictive model, based on the AI, in particular on the Machine Learning, for the automated recognizing and counting of helminth eggs. This model is a variant of a method for detecting objects in images using a single deep neural network, named Single Shot Multibox Detector (SSD) (Liu et al., 2016). The model optimizes the detection activity using convolutional network layers that produce bounding boxes (with coordinates inside the picture) as showed in Figure 3.11.

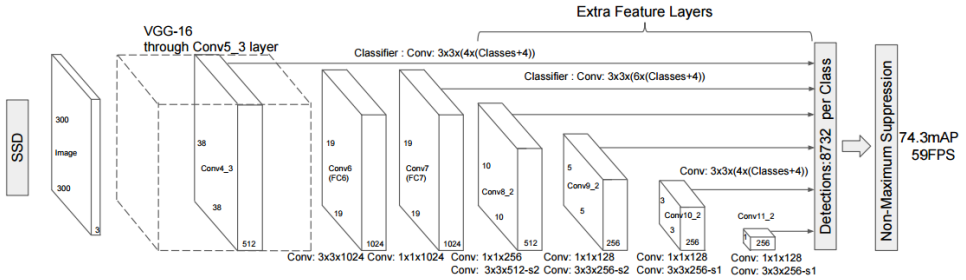


Figure 3.11. Convolutional network used by the SSD model. A VGG-16 network is used as a base, and convolutional feature layers decrease in size progressively in order to allow predictions of detections at multiple scales.

3.5.2 Software training

Up to date, to train the software, a dataset with 11.136 objects was used including samples of GIN from large and small ruminant faeces (confirmed by experts). The acquired images are first segmented into background and objects, which can be either impurities or pseudo-parasites through the use of ImageJ (Italian National Institute of Health) with the main goal of being able to characterize the parasite species (Figure 3.12) and to automatically count eggs (Grishagin, 2015).

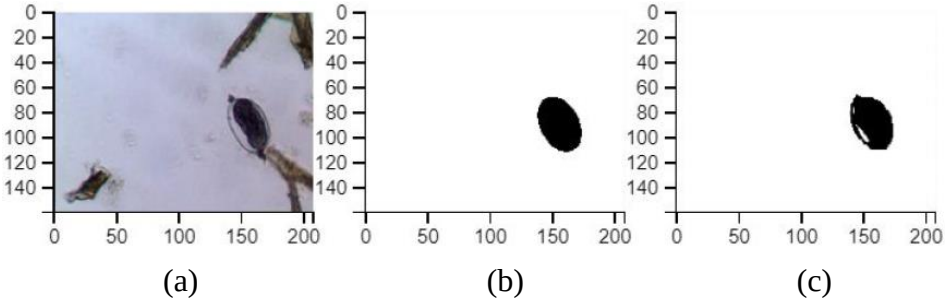


Figure 3.12 (a) A GIN egg image (b) A segmentation using ImageJ (c) The same image recognized by the AI software.

Up to date, the KFM can be used as a semi-automated system by a technician using the macro and micro-movement through the app that connect automatically the software to the mobile devices. The development of the AI predictive model will be very useful for an easy-to-use, low cost and precise automated system for the reading and counting of PEs allowing a

rapid assessment of FEC/FECR to assist the new generation of operators (i.e. technicians, physiscians, veterinarians, farmers) in veterinary and human field.

In conclusion, the KFM is a promising system that will allow to overcome limitations of FEC techniques. Validation studies are in progress in veterinary and human field and the first results will be published in a short time.

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CHAPTER IV
General discussion

4.1 General discussion

In this PhD tesis, after an overview of the most common copromicroscopic techniques to detect and count the gastrointestinal nematodes (GIN) in ruminants (CHAPTER I), insights on faecal egg count (FEC) and faecal egg count reduction (FECR) techniques in sheep and cattle were reported in CHAPTER II. The results obtained showed that: i) the Mini-FLOTAC was more sensitive, precise and accurate of Cornell-Wisconsin and McMaster technique for the FEC/FECRT of GIN in sheep and cattle, but also in horses, as described in CHAPTER II (studies 1.0 and 3.0); ii) the pooling strategy and the use of portable FEC-kits *on-farm* are rapid, cost-effective, and reliable procedures for the assessment of GIN egg excretion and can be used cautiously for assessing the intensity of infection of GIN and anthelmintic activity in cattle (CHAPTER II, study 2.0); iii) the standardization of FEC techniques, including the preservation of sample and the flotation solution to use, is very important to obtain reliable and reproducible results. In fact, the use of the same Standard Operationg Procedures (SOPs) permitted to obtain not statistically different recovery rates values of GIN using Mini-FLOTAC in Belgium and in Italy laboratories (CHAPTER II, study 3.0).
However, the diagnostic performances of a FEC technique can be strongly influenced also by operator dependent variations. Therefore, the development of automated diagnostic techniques can be an effective resolution to reduce human errors and time of analysis. Although many authors tried to develop new tools, as reported in CHAPTER III, these prototypes showed some limitations. To automate the FLOTAC and Mini-FLOTAC techniques, a digital microscope, the Kubic FLOTAC microscope (KFM), has been developed and a firstvalidation to detect GIN eggs in cattle has been performed. The KFM is based on the use of sensitive, accurate, precise and standardized techniques, FLOTAC and Mini-FLOTAC combined with a this new automated microscope, resulting a promising tool for FEC/FECR (on lab and in-field) (CHAPTER III).

In the next paragraphs, important updates and insights obtained in this thesis will be discussed extensively.

4.1.2 The importance of using the Mini-FLOTAC technique for the detection of GIN infections

The importance to use a technique with high diagnostical and technical performance for GIN to obtain reliable results for FEC/FECR in ruminants is very important, as discussed also in the CHAPTER I. In particular, the CHAPTER II, study 1.0 and CHAPTER II, study 3.0 provide new insights toward the evaluation of Mini-FLOTAC performances for the detection of GIN in ruminants. In CHAPTER II, study 1.0 an evaluation of sensitivity, accuracy and precision of the Mini-FLOTAC, McMaster and Cornell-Wisconsin techniques for detection of GIN in small ruminants and horses was performed. Eggs extracted from equine and ovine faeces, in known numbers, have been added to equine and ovine negative faeces, in order to obtain four levels of contamination: 10, 50, 200 and 500 eggs per gram (EPG) of faeces. The results showed that the Mini-FLOTAC is the most sensitive, precise and accurate technique at all levels of contamination. McMaster showed 100% sensitivity only at concentrations > 200 EPG, whilst Cornell-Wisconsin significantly underestimated EPG at all levels of contamination. These results are in agreement with those obtained from other comparisons with Cornell-Wisconsin, McMaster and FECPAK for FEC and FECRT of GINs in sheep (Rinaldi et al., 2014; Godber et al., 2015; Kenyon et al., 2016; Paras et al., 2018; Bosco et al., 2018). Moreover, a different percentage of egg recovery was detected between ovine and equine faeces. In fact, when eggs of GINs isolated from positive ovine faeces were add to negative ovine faeces, the recovery percentage was 100%, whilst when eggs isolated from ovine faeces were added to equine faeces, the recovery rate was 91.0%. This difference could be due to the different consistency of the faeces of the two hosts; the horse's faeces, in fact, have a high presence of fibers which could affect the recovery of the eggs.
In CHAPTER II, study 3.0, the sensitivity, accuracy and precision of the Mini-FLOTAC and McMaster techniques (at two different reading levels, grids and chambers) for the diagnosis of GIN in cattle was assessed. Negative cattle faeces contaminated with five levels of GIN eggs were used: 10, 50, 100, 200 and 500 UPG. The Mini-FLOTAC technique was the most sensitive, accurate and precise technique at all levels of contamination.
Therefore, the Mini-FLOTAC provides reliable results and can be a good candidate to perform a standardized FEC/FECR of GIN in ruminants on-farm as well as in the laboratory as demonstrated previously also by other authors (Dias de Castro et al., 2017; George et al., 2017; Paras et al., 2018).

4.1.3 The importance of pooling strategy and portable FEC-kits for the assessment of GIN infections

In order to reduce cost, time commitment and workload for FEC/FECR, pooling strategy resulted very efficient in sheep (Baldock et al., 1990; Nicholls et al., 1994; Morgan et al., 2005; Rinaldi et al., 2014; Kenyon et al., 2016) and in cattle (Ward et al., 1997; George et al., 2017). Mean individual and pooled counts showed similar results. However, the previous two studies performed in cattle were based on a composite sample made from two pools of five individual faecal samples or on a single pool of all the individual samples and did not investigate the effect of different pool sizes on the FEC estimation. Therefore, in the CHAPTER II, study 2.0 an evaluation of FEC/FECR, using different pool sizes, i.e. pool of 5 individual samples, pool of 10 individual samples, pool of all animals (global pool) in cattle farm, was performed in Italy and in France, showing that this approach is valid, mainly to evaluate FEC before treatment. Moreover, a portable FEC-kit (described in CHAPTER I, Figure 1.9) to perform pooled FEC on-farm was validated using a portable microscope with batteries (Celestron, USA) (Figure 4.1).

Figure 4.1 The portable FEC-kit to performed analysis on field

Therefore, the obtained results highlight that the combination of pooling strategy and the use of a portable FEC-kit on-farm is a rapid and cost effective strategy to assess infection intensity, before and after anthelmintic treatment.

4.1.4 The importance of standardization of the copromicroscopic techniques

In CHAPTER I different copromicroscopic techniques are reported. Unfortunately, diagnostic and research laboratories around the world use different protocols of FEC techniques for their activities. Therefore, it's very important to establish precise standard operating procedures (SOPs) for FEC techniques, including preservation of samples before analysis and the flotation solution to use. Besides, findings of the study in CHAPTER II, study 3.0 highlight this priority. Using cattle spiked faecal samples at five levels of GIN eggs contamination (10, 50, 100, 200 and 500 EPG), was evaluated the sensitivity, accuracy and precision of Mini-FLOTAC and McMaster (at two levels of reading: grids and chambers) techniques, as well as their reproducibility in two laboratories, one in Belgium and one in Italy. Results showed that Mini-FLOTAC gave a higher percentage of recovery of GIN eggs than McMaster at both two reading levels and for all the levels of contamination, in Italy and in Belgium. Moreover, Mini-FLOTAC showed a higher sensitivity and precision, without statistically significant differences between laboratories in two countries. Therefore, these results showed that if Standard Operating Procedures (SOP) are used, the reproducibility of techniques is high. Many factors can influence the technical variability of FEC techniques; the main are the faecal preservation (typology of fixative and duration of preservation) before the analysis and the choice of the flotation solution. The incorrect preservation of the faecal sample can cause a significant reduction in the number of GIN eggs, e.g. the freezing destroys the eggs (Rinaldi et al., 2011). To avoid the reduction of the GIN eggs number, refrigeration of the faecal samples at +4°C up to three days or the vacuum packing at +4 ◦C up to 21 days permits a good storage (Rinaldi et al., 2011). Moreover, as yet discussed in CHAPTER I, the standardization of flotation solution (FS) has an important role for FEC techniques. In the CHAPTER IIthe findings of the, study 3.0 showed, one more time, that the FSs might influence the performance of the technique and therefore its precision and accuracy (Cringoli et al., 2017). In fact, in different studies it has been shown that sodium chloride (specific gravity = 1.200) was the best flotation solution for GIN FEC and it is recommended for GIN detection by Mini-FLOTAC (Cringoli et al., 2017). In this study 3.0 a recovery rate of 98.1% of GIN in cattle was found, according to the recovery rate of 100% for GIN in sheep as reported in the study 1.0 described in CHAPTER II. Therefore, the low recovery rates found in other studies could be due to the inappropriateness of the FSs (i.e. sodium nitrate

with a specific gravity=1.25-1.30 (Paras et al., 2018); glucose-NaCl flotation medium with a specific gravity= 1.24-1.28 (Noel et al., 2017; Nàpravnikova et al., 2017; Scare et al., 2017).
Research priorities should include the development of more standardized and less labour intensive systems for parasite egg counts for both pen-side and laboratory use (Rinaldi et al., 2019), including methods of automated sample processing and image analysis, as indicated in the STAR-IDAZ (https://www.star-idaz.net) diagnostic road map for research on helminths and anthelmintic resistance.

4.1.5 Towards the development of a FLOTAC and Mini-FLOTAC automated system for parasitological diagnosis in animals: the Kubic FLOTAC Microscope

The development of an automated system for the analysis of FEC/FECR has an important implication towards the achievement of a reliable diagnosis of GIN in ruminants. In CHAPTER III the Kubic FLOTAC Microscope (KFM) was presented. The main advantage of this automated microscope is the combination of the sensitive, accurate, precise and standardized diagnostic techniques, FLOTAC and Mini-FLOTAC, with a reliable system to capture and analyse images that permits to reduce human errors and time of reading. Moreover, the high quality of obtained images will be fundamental to develop a software, based on Artificial Intelligence (AI) for the automated recognizing and counting of GIN eggs. This new tool might be used: i) in laboratories or directly in field; ii) by any operator without a specific training (the pictures can be sent by internet to references laboratories); iii) without requiring special equipment; iv) without requiring high cost.

4.2 Future Perspectives

In order to going on our main research goal to improve FLOTAC and Mini-FLOTAC techiques, it's important to note that further studies are required to validate a software, based on AI, also for other parasitic elements (PE) of veterinary and human importance. Moreover our final aim will be to automate all steps of FLOTAC and Mini-FLOTAC techniques, in order to

obtain a fully automated process,from the sampling to the reading of parasitic elements (Pes) (Figure 4.2).

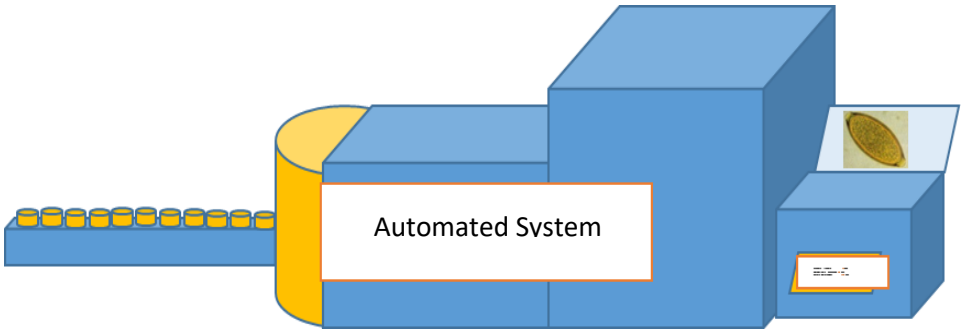

Figure 4.2 Diagram of the fully automated system for the copromicroscopic diagnosis with FLOTAC and Mini-FLOTAC tecniques.

In conclusion, this PhD thesis showed that this new system is promising to overcome gaps and limitations of FEC techniques through the creation of new data streams from high-throughput diagnostic tests.

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