

**PREVALENCE OF GASTRO-INTESTINAL PARASITES OF LIVESTOCK AND
DOGS AND RISK FACTORS FOR TRANSMISSION WITH EMPHASIS ON
GIARDIA AND *CRYPTOSPORIDIUM* IN MAGUDE DISTRICT, MAPUTO
PROVINCE, MOZAMBIQUE**

By

Regina Daniel Miambo

215076147

regil.miambo@gmail.com, r.miambo@yahoo.com.br

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School of Life Science

College of Agriculture Engineering and Science

University of KwaZulu-Natal

Durban, 400

Supervisor: Prof. Samson Mukaratirwa

Co-Supervisors: Dr. Alberto Pondja Junior

Prof. Johan Lindh

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ABSTRACT

Objectives: The main objective of this study was to determine the prevalence of gastrointestinal parasites and risk factors for transmission of *Giardia* and *Cryptosporidium* in livestock and dogs of Magude District, Maputo, Mozambique.

Methods: A total of 696 faecal samples (480 from calves, 60 from goats and 156 from dogs, between 0 and 7 months) were randomly collected from the rectum of animals of both sexes from February to September, 2015. Willis and McMaster methods using NaCl solution were applied in all faecal samples to identify and quantify gastrointestinal helminthic and protozoal infections. To improve the sensitivity of the tests in detection of *Giardia* and *Cryptosporidium*, the formol-ether method was applied and the sediment obtained was used for the modified Ziehl Neelsen (mZN) for detection of *Cryptosporidium* oocysts, direct immunofluorescence (DIF) and indirect immunofluorescence (IIF) tests for both *Cryptosporidium* oocysts and *Giardia* cysts. Since the secondary antibody for IIF was derived from goats, this method was not applied in goat samples. To determine the risk factors, a questionnaire was administered to dog owners and livestock farmers. Odds ratios (OR) and 95% confidence interval (CI), Chi Square or Fisher exact test for risk factors and general linear model multivariate for differences between localities of Magude District were applied using SPSS programme and $p < 0.05$ was considered to be statistically significant. The sensitivity and specificity of mZN and IIF were determined using MedCalc software with DIF test as gold standard.

Results: Using Willis, IIF and DIF, the prevalence of *Giardia* in calves was 0%, 8.1%, and 6.0%, in dogs 0.6%, 8.3% and 5.7% and for goats it was 0% and 13.3% respectively and the IIF was not done. The prevalence of *Cryptosporidium* in calves using Willis, mZN, IIF and DIF was 0%, 3.8%, 4.7% and 0.4% in dogs it was 0%, 0.6%, 6.4% and 0.6% respectively and in goats was 0% for all tests. All positive samples to DIF, IIF and mZN were negative by PCR. Additionally, the parasites and prevalence detected in dogs were *Sarcocystis* spp. (3.8%), *Isospora* spp. (2.6%), *Ancylostoma* spp. (60.3%), *Toxocara canis* (5.8%), Taeniidae (1.9%), *Trichuris vulpis* (1.3%), *Spirocerca lupi* (0.6%); in calves and goats, Strongylid (50.8%, 31.6%), *Eimeria* spp. (17.5%, 41.6%) and *Moniezia* spp. (3.3% and 11.6%) respectively.

The mZN test showed high sensitivity (100%) and specificity (96.2% and 100%) in detecting *Cryptosporidium* oocysts. The sensitivity and specificity of IIF test to detect both parasites was also high. The sensitivity ranged between 88.9% and 100%, specificity between 95.4% and 98.5% for *Giardia* and 100% of sensitivity, 93.2% and 93.9% of specificity for *Cryptosporidium*. In contrast, the Willis lacked sensitivity for *Giardia* and *Cryptosporidium*

infections (0%). The lack of regular treatment against parasitic infections in calves and the source of water (mostly the river) were identified as a risk factor in the transmission of *Giardia* and *Cryptosporidium* in calves and dogs ($p < 0.05$).

Conclusion: *Giardia* and *Cryptosporidium* are prevalent in Magude District, although the risk of zoonotic transmission through molecular technique was not done due to low numbers of oocysts/cysts in the positive samples. The main helminthic parasites detected through floatation technique for dogs were *Toxocara canis* and *Ancylostoma* spp., for cattle and goats were strongylids and *Eimeria* spp. and the intensity of infection was low.

PREFACE

The research contained in this thesis was completed by the candidate, from February 2015 to May 2016, while based in the Discipline of Parasitology, School of Life Science, University of KwaZulu-Natal, Westville Campus, under the supervision of Professor Samson Mukaratirwa and Co-supervision of Doctor Alberto Pondja Junior and Professor Johan Lindh. The research was supported by UEM/ASDI “Impact of Zoonotic Diseases on Public Health and Animal production in Mozambique”.

The contents of this study represent original work by the author, and have not been submitted in any form to another tertiary institution, except where the work of others is acknowledged in the text.

Signature_____

Signed: Prof. Samson Mukaratirwa

Date:_____

DECLARATION

I Regina Daniel Miambo, declare that:

1. The research reported in this thesis, except where otherwise indicated, and is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Signature: _____

Signed: Regina Daniel Miambo

Date: _____

I dedicate this thesis to my shining star in the sky, my late husband, Gilberto Boaventura

Januário "*in memory*"

"When doubts filled my mind, your comfort gave me renewed hope and cheer"

Psalms 94:19

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LIST OF SYMBOLS

%: Percentage

°C: Degrees Celsius

µl: microlitre

µm: micrometre

g: gram

kg: kilogram

mg: milligram

ml: millilitre

mm: millimetre

β: Beta

LIST OF ABBREVIATIONS

BSA: Bovine Serum Albumin

CI: Confidence Intervals

CLM: Cutaneous Larva Migrans

Cowp: *Cryptosporidium* oocysts wall protein

CPG: Cysts per gram of faeces

d: Precision measured

DIF: Direct Immunofluorescence

DNSV: Direcção Nacional dos Serviços de Veterinária

ELISA: Enzyme-Linked Immunosorbent Assay

EPG: Eggs per gram of faeces

FITC: Fluorescein Isothiocyanate

GI: Gastrointestinal

GIP: Gastrointestinal Parasites

gp: glycoprotein

HIV: Human Immunodeficiency Virus

hsp: heat shock protein

IFA: Immunofluorescence Assay

IgA: Immunoglobulin A

IIF: Indirect Immunofluorescence

INE: Instituto Nacional de Estatística

L1: First stage larva

L2: Second stage larva

L3: Third stage larva

mAb: monoclonal antibody

MEA: Ministério da Administração Estatal

mZN: modified Ziehl Neelsen

N: Population size

n: sample size

np: number of positives

NaCl: Sodium Chloride

OPG: Oocysts per gram of faeces

OR: Odds ratio

pAb: Polyclonal antibody

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

Pexp: Expected Prevalence

RFLP: Restriction Fragment Length Polymorphism

RNA: Ribonucleic Acid

rpm: revolutions per minute

syn: synonym

UEM: Universidade Eduardo Mondlane

USA: United States of America

VLM: Visceral Larva Migrans

VSPs: Variant Specific Proteins

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1. INTRODUCTION

1.1. Background

In recent decades the food-borne and water-borne pathogens have been of concern in public health, because of their involvement in diarrhoea outbreaks in humans, livestock and companion animals (Paraud and Chartier, 2012) especially in developing countries with poor sanitation (Santín *et al.*, 2007).

Among these groups of pathogens, protozoans of the genus *Giardia* and *Cryptosporidium* are of public health importance (Monis and Thompson, 2003) being also associated with opportunistic infections in immunocompromised patients (Dawson, 2005). More than six species of *Giardia* and ten species of *Cryptosporidium* have been described, however, only one from each genus is considered to be of medical importance (*Giardia duodenalis* and *Cryptosporidium parvum*) and multiple genotypes have been reported, some of them are of zoonotic potential (Thompson *et al.*, 2008; Xiao and Fayer, 2008). Giardiasis and cryptosporidiosis are major causes of morbidity in young animals, and neonates with consequent economic loss (De Graaf *et al.*, 1999) and are associated with different levels of mortality particularly when they occur in concomitant infections generally with helminthic parasites belonging to different groups (Taylor *et al.*, 2007).

In the group of domestic animals with close contact with humans, special attention is given to dogs because they are host to a wide diversity of gastrointestinal parasites (GIP) known to be infective to humans (Robertson and Thompson, 2002) and the nematode parasites of genus *Toxocara canis* and *Ancylostoma* spp. are mostly described as causing pathologic conditions in humans such as Visceral Larva Migrant (VLM) and Cutaneous Larva Migrant (CLM) respectively. These are often associated with contamination of public or recreation places by dogs faeces containing eggs or larva of parasites (Gingrich *et al.*, 2010; Soriano *et al.*, 2010). In addition to the referred parasite, dogs also play an important role in the maintenance of transmission of *Echinococcus* species which causes pathological effects in humans mainly associated with the growing site of larval form (Magambo *et al.*, 1999).

In goats and cattle, the main gastrointestinal (GI) helminths are derived from infections by nematode parasites of different genus belonging mainly to order Strongylida. Different studies in the occurrence of GI parasites in ruminants have shown high prevalence of infections by different species of nematodes in association with coccidian of the genus *Eimeria* (Anene *et al.*, 1994; Mbuh *et al.*, 2008; Gwazea *et al.*, 2009; Sultan *et al.*, 2016), which may exacerbate

the clinical status of infected animal if pathogenic species are involved since both are occasionally related with cases of diarrhoea (Taylor *et al.*, 2007). In Mozambique no information is available regarding the prevalence of gastrointestinal parasites of cattle, goats and dogs with emphasis on *Giardia* and *Cryptosporidium* spp., especially in Magude District which is the second largest producer of cattle and other livestock in the Maputo Province of Mozambique. Molecular characterization of parasite *Giardia* and *Cryptosporidium* in this region is of great importance in order to determine the epidemiology, host range and risk factors for transmission providing the basis for better understanding of the links between infections in humans and animals, genotypes or subtypes involved and their zoonotic potential (Thompson *et al.*, 2008).

1.2.Objectives

The study was aimed at determining the prevalence of gastrointestinal parasites of cattle, goats and dogs in Magude District with particular emphasis on *Giardia* and *Cryptosporidium* spp. using different diagnostic techniques

1.2.1. Specific objectives

- To determine gastrointestinal parasites infecting cattle, goats and dogs in Magude District
- To identify genotypes of *Giardia* and *Cryptosporidium* species in livestock and dogs in Magude District and their zoonotic significance
- To determine the risk factors associated with transmission of *Giardia* and *Cryptosporidium* spp. between animals and humans in Magude District
- To determine the sensitivity and specificity of modified Ziehl Neelsen and Indirect Immunofluorescence tests in the diagnosis of *Giardia* and *Cryptosporidium*

2. LITERATURE REVIEW

2.1.ZOONOTIC HELMINTHS OF DOGS

Ancylostoma spp. and *Toxocara* spp. are cosmopolitan parasites both belonging to class nematode and distinct Superfamily (Ancylostomatoidea and Ascaridoidea respectively). Adults inhabit the small intestine of domestic and wild dogs, developing by direct life cycle on which the third stage larvae (L3) and egg containing the second stage larvae (L2) respectively are described as infective to susceptible hosts (Taylor *et al.*, 2007).

Three species are described, *Ancylostoma tubaeforme*, *A. caninum* and *A. braziliense*, but only the last two can infect humans causing a pathological condition called Cutaneous Larva Migrant (CLM) derived from the location of L3 on the skin (Urquhart *et al.* 1998). Humans can also acquire infection by *Toxocara canis* ingesting embryonated eggs and the larvae migrate and encyst in different organs producing the syndrome named Visceral Larva Migrans (VLM) or ocular if the larva is located in the eye globe (Macpherson, 2013). In dogs two main routes of infection are common for both parasites, the ingestion of infective forms from the environment and transmammary route (related with the latency of larva). The percutaneous and pre-natal are related to *Ancylostoma* and *Toxocara* infections respectively. The infection by *Toxocara* is followed by migration of larval stage through the liver and lungs (animals below 3 months of age) or through different organs (somatic migration in animals above 3 months) where they encyst (Taylor *et al.*, 2007).

Symptoms of infection by *Toxocara* are related with migration of larva in lungs and massive presence of adults on intestines, cough and diarrhoea are mostly observed. *Ancylostoma* spp. is a hematophagous parasite, described as the main cause of acute anaemia sometimes with bloody diarrhoea mainly in animals up to 3 weeks (Urquhart *et al.*, 1998).

Different species of Taeniidae with zoonotic potential can infect dogs (definitive host) but great importance has been given to *Echinococcus granulosus* and *Echinococcus multilocularis* which cause cystic echinococcosis and alveolar echinococcosis respectively in humans, with predilection for growth in the liver and lungs (Magambo *et al.*, 1999). Information about the occurrence of *Ancylostoma* spp. and *Toxocara canis* in Mozambique was referred by Cruz and Siva (1971) and Santos *et al.* (2013).

2.2. HELMINTHS AND COCCIDIA IN GOATS AND CATTLE

In ruminants the GIP are indicated as one of the main constraints due to the cost with treatment, low weight gains and deaths, occurring mainly in free-range production where animals of different farms share the same grazing areas (Sultan *et al.*, 2016).

In the nematode group, species of superfamily Trichostrongyloidea (*Haemonchus* spp., *Trichostrongylus* spp., *Ostertagia* spp.) are referred to as significant pathogens due to the abundance related to the high fecundity of females and the pathogenic effects on the host (Urquhart *et al.*, 1998). The severity varies according to the intensity of infection, the genus and pathogenicity of parasite involved, the immunological and nutritional state of host (Houdijk *et al.*, 2012). Diarrhoea, inappetence and loss of weight are mainly observed with exception of *Haemonchus* spp. which is indicated as the primary cause of death of animals by anaemia (Taylor *et al.*, 2007).

The life cycle is direct, generally animals acquire infection by ingestion of third stage larvae (L3) during the grazing, adult males and females are located in different segments of the GI tract depending on the predilection of parasite. Typical eggs of Strongylid are eliminated together with the faeces of infected animal and the developing from first stage larvae (L1) to the third stage (L3) occur in the environment (Urquhart *et al.*, 1998).

Cestoda of genus *Moniezia* (Family Anoplocephalidae and order Cyclophyllidea) are mostly reported in small ruminants and occasionally in cattle with infections usually asymptomatic, unless the amount of adults in the intestines is high enough to block the passage of food and consequently the absorption of nutrients. The prevalence and distribution is related to the distribution of Oribatidae forage mites which are the intermediate hosts (Taylor *et al.*, 2007).

In addition to the above helminths, cases of diarrhoea sometimes bloody especially in calves and young goats can also be related to infections by *Eimeria* species, this protozoan parasite belongs to the phylum Apicomplexa, order Eucoccidiida and different species with different levels pathogenicity and host specificity can be identified in a faecal sample (Andrews, 2013). The life cycle is monoxenous, animals get infected by ingesting sporulated oocysts in the water or food. In the host intestinal cells the parasitic forms reproduce by schizogony and gametogony culminating with the formation of non sporulated oocysts which are eliminated with the faeces which will be reproduced by sporogony under optimal conditions of temperature, humidity and oxygen (Taylor *et al.*, 2007).

Sometimes signs of diarrhoea are absent in infections by *Eimeria* spp. and usually low weight gains due to reduction of the food intake are observed, which might be exacerbated by

depreciation of immunological system of the animal with the invasion of nematode helminths (Andrews, 2013).

Clinical signs of helminth GIP infections are not pathognomonic and may induce to inappropriate and sometimes unnecessary treatment (low dose or successive administrations of the same drug) and the development of anti-helminthic resistance if effective diagnosis with the identification of the parasites involved is not instituted (Torres-Acosta and Hoste, 2008; Jackson *et al.*, 2012).

In Mozambique little information is available regarding helminth GIP of cattle and small ruminants. In cattle Cruz and Siva (1971) reported the following parasites, *Trichostrongylus axei*, *Haemonchus placei*, *Cooperia* spp., *Oesophagostomum radiatum*, *Bunostomum phlebotomum*, *Neoascaris vitulorum*, *Paramphistomum microbothrium* and *Moniezia benedeni*. Specht (1982) reported the seasonal incidence of the following nematode parasites in sheep and goats from south of Mozambique (*Trichostrongylus colubriformis*, *Cooperia* spp., *Oesophagostomum columbianum*, *Trichuris* spp., *Strongyloides papillosus*, *Moniezia* spp., and *Paramphistomum* spp.).

2.3. GIARDIA

2.3.1. Taxonomy and characteristics

Giardia spp. is an intestinal flagellate grouped in the phylum Protozoa, subphylum Sarcomastigophora, class Mastigophora, order Diplomonadida, family Hexamitidae (Taylor *et al.*, 2007).

There are two developmental stages in the life cycle which are the trophozoite (motile vegetative form) and cyst (resistant infective form) (Adam, 2001; Thompson, 2004; Dawson, 2005). The trophozoite has a pyriform to ellipsoidal form, bilaterally symmetrical body with convex dorsal side and a large sucking disk on the ventral side; four pair of flagella, two anterior nuclei and pair of median body; being approximately 12–15 µm long and 5–9 µm wide (Taylor *et al.*, 2007; Adam, 2014) (Figure 1). The cyst is ovoid with four nuclei and 8–12 µm × 7–10 µm in size (Taylor *et al.*, 2007).

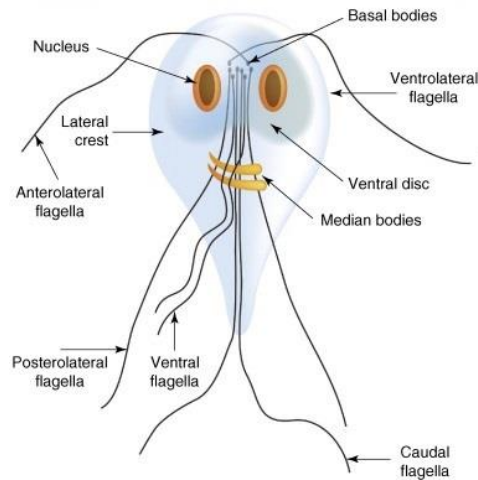


Figure 1: Morphology of *Giardia* trophozoite (Monis *et al.*, 2009)

2.3.2. History

Examining his own diarrhoeal faeces, Antony Van Leeuwenhoek observed for the first time the flagellate protozoa by light microscope in 1681 (Adam, 2014). Over time this parasite received several names: *Cercomonas* (Lambl in 1859), *Lamblia intestinalis* (Blanchard in 1888), *Giardia duodenalis* (Stiles in 1902), *Giardia lamblia* and *Giardia enterica* by Kofoid and Christiansen in 1915 and 1920 respectively (Adam, 2001).

2.3.3. Species and assemblages of *Giardia*

Most of the *Giardia* species were initially described based on host specificity, thus more than 40 species were identified (Conboy, 1997), and later with detailed description of morphological characteristics the number of species decreased to 6 (Adam, 2001; Thompson *et al.*, 2008; Robertson, 2014) and the following species have been described; *Giardia agilis* from amphibians, *Giardia muris* from rodents (Adam, 2014) birds and reptiles (Dawson, 2005), *Giardia ardeae* and *Giardia psittaci* from birds, *Giardia microti* from rodents (Karanis, 2011; Adam, 2014) and the most important, *Giardia duodenalis* (syn. *Giardia lamblia* and *Giardia intestinalis*) from wild and domestic mammals including humans (Taylor *et al.* 2007).

Observations of these species under light microscope, show similar morphology but using molecular techniques they do show marked genetic differences among isolates from different host species (Adam, 2001; Thompson, 2004; Hunter and Thompson, 2005; Thompson *et al.*, 2008). Molecular sequencing of *Giardia duodenalis* has been done based on glutamate dehydrogenase/ *gdh*, triosephosphate isomerase/ *tpi*, and β giardin/ *bg* genes (Plutzer *et al.*,

2010; Feng and Xiao, 2011) and grouped this specie into eight (A to G) genotypes (Itagaki *et al.*, 2005 and Santín *et al.*, 2007) and H (Feng and Xiao, 2011). To date, only genotypes A and B are known to be of zoonotic importance (Adam, 2014), affecting domestic dogs, cats, livestock and wild animals (Fayer *et al.*, 2004; Trout *et al.* 2006). In turn these two genotypes have been subdivided into A I (the zoonotic one), A II (humans); B III and IV, both specifically in humans (Thompson, 2004). Assemblage C and D have been reported in canids isolates (Smith *et al.*, 2007); assemblage E in hoofed stock (cattle, sheep, goats, pigs, horses); assemblages F in cats and G in rat isolates (Fayer *et al.*, 2004). The new group is assemblage H, recently reported in seals (Feng and Xiao, 2011).

2.3.4. Life cycle

The life cycle of *Giardia* starts when a susceptible host ingests the cystic forms (A) in the environment through contaminated water, food (Dawson, 2005) or by direct faecal-oral contact (Adam, 2001). The excystation occurs in the acid environment of stomach followed by entry into the duodenum (B) where the trophozoite replicates by binary fission (C) producing two trophozoites from each (Taylor *et al.*, 2007); immediately the encystation (D) occurs stimulated by bile salts exposure and cholesterol starvation (Dawson, 2005; Feng and Xiao, 2011; Adam, 2014) forming cysts (E) in the jejunum which can be passed in formed faeces. Trophozoites are normally detected in diarrheic faeces, although because of their fragility, they may survive only for short periods in the environmental (Conboy, 1997).

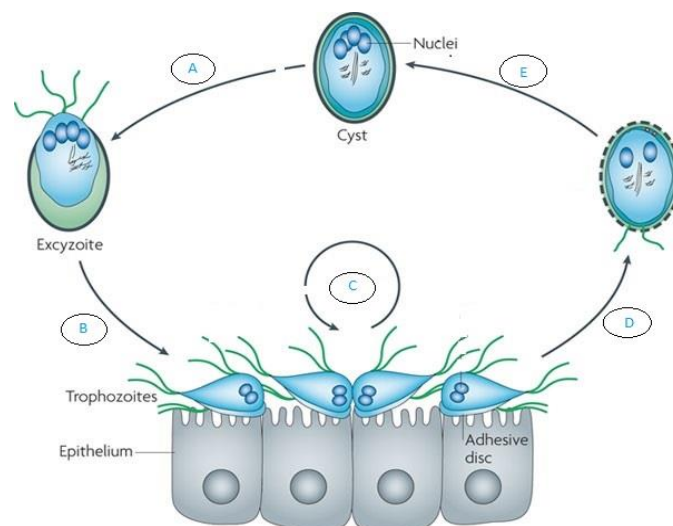


Figure 2: *Giardia* life cycle, adapted from Ankarklev *et al.* (2010)

2.3.5. Pathogenesis and clinical manifestation

The severity of infection depends on factors related to the host (the immune or nutritional status and age of infected host) or related to the parasite (virulence and pathogenicity of strain) (Olson *et al.*, 2000; Cotton *et al.*, 2011).

In the proximal small intestine, the trophozoites attach to the enterocytes where they get nutrients causing malabsorption by mechanical blockage and diarrhoea by damage of mucous membrane (Dawson, 2005; Adam, 2014). Infections with *Giardia* spp. commonly induces immune responses with primarily production of anti-parasite IgA (Solaymani-Mohammadi and Singer, 2010) and subsequent apoptosis with increase of epithelial permeability (Adam, 2014) induced by disruption of apical junctional complex proteins (zonula occludin-1). Infection can vary from acute to chronic, although some hosts may be asymptomatic (Robertson *et al.*, 2010). When disease occur, the most common sign is continuous or intermittent diarrhoea accompanied by weight loss and lethargy (Taylor *et al.*, 2007), young animals and children are more susceptible (Conboy, 1997).

The adaptability of *Giardia* species to different intestinal environments and the course of infection is determined by antigenic variation afforded by approximately 300 cysteine-rich proteins, called variant specific proteins (VSPs) where in the course of infection, existing proteins are replaced by resistant proteins to antibodies produced by exposure of the previous (Adam, 2014). There is evidence of resistance in individuals already exposed to the parasite without developing clinical symptoms (Adam, 2014).

2.3.6. Epidemiology

In the epidemiology of *Giardia* spp. it is important to understand the potential for cross-species transmission, the genotypes and risk factors involved (Feng and Xiao, 2011). The parasite is maintained by one of the following important cycles: between animals and humans; from animal to animal, human to human, by waterborne or foodborne transmission (Thompson, 2004). Young animals are considered to be most important source of infection because they can excrete high number of cysts (10^6 cysts per gram of faeces) than older animals (1-10 cysts per gram of faeces) (Geurden *et al.*, 2010). Low doses are needed for infection (10-100 cysts) which are immediately infective after elimination (Adam, 2014).

Giardia duodenalis has a world-wide distribution with variations in prevalence between developed and developing countries (Adam, 2014). Surveys on molecular characterization of *Giardia* strains in domestic animals have been reported in many countries in Africa like Tanzania (Di Cristanziano *et al.*, 2014); Egypt (Helmy *et al.*, 2013; Foronda *et al.*, 2008);

Ethiopia (Gelanew *et al.*, 2007) in which assemblages with zoonotic potential were found. The prevalence varies between countries, animal species of study and method of diagnosis used ranging from 56.8% in dogs (Traub *et al.*, 2009) 25.5% in lambs; 35.8% (Geurden *et al.*, 2008a), 38% (Castro-Hermida *et al.*, 2005) in goats kids and 49% in calves (Hannes *et al.*, 2006).

2.3.7. Treatment and control

Different drugs of the benzimidazole group such as albendazole and fenbendazole and also paramomycine are effective in the treatment of giardiasis in calves. In dogs nitromidazole drugs (metronidazole, tinidazole) (Taylor *et al.*, 2007) and secnidazole in cats (Da Silva *et al.*, 2011) are also effective.

In the control of giardiasis we need to take into account the capacity of resistance of cystic forms in the environment. When exposed to chlorine, in cool or damp environment the cyst can remain infectious for more than one month (Dawson, 2005). A *Giardia* vaccine consisting of a lyophilized trophozoite of four parasite strains for prevention of clinical signs and reduction of cyst shedding in dogs and cats reducing the contamination of environment is available in the USA (Olson *et al.*, 2000).

2.4. CRYPTOSPORIDIUM

2.4.1. Background

In 1907, Ernest Edward Tyzzer, the pioneer of *Cryptosporidium*, was the first one isolating oocysts of this parasite from gastric glands of mice naming it *Cryptosporidium muris*. After five years he isolated the same parasite from small intestine of mice, however smaller than the first one, naming *Cryptosporidium parvum* (Chalmers, 2014). For long there was no interest in studying the pathogenicity of this parasite until 1970s when it was linked to outbreaks of diarrhoea in cattle (Dixon, 2014) and in immunocompromised patients (Kosek *et al.*, 2001) since there different studies have shown that there is biological and genetic differences between *Cryptosporidium* spp. in different host species (Karanis, 2011).

2.4.2. Characteristics

Cryptosporidium spp. belong to phylum Protozoa, subphylum Apicomplexa (Sporozoa), subclass Coccidia, order Eucoccidiida (Eucoccidiorida), suborder Eimeriina (Eimeriorina), and family Cryptosporidiidae (Plutzer and Karanis, 2009). Mature oocysts are thick walled (Taylor *et al.*, 2007), ovoid or spheroid, 4.6 μm –5.4 μm \times 3.8 μm –4.7 μm (mean

5.0 μm \times 4.5 μm), containing four sporozoites (Fayer *et al.*, 2000). They are resistant surviving for many weeks under cool and moist conditions (De Waal, 2012; Dixon, 2014).

2.4.3. Species and genotypes

Initially it was considered that infections with *Cryptosporidium* were species specific, in which most of species were named based on morphological structure of the oocysts and this created confusion in the identification due to similarity of oocysts and their small size (Fayer *et al.*, 2000). Based on morphologic and host specificity criteria (Monis and Thompson, 2003) more than 20 species have been described and with the advent of molecular techniques for identification, about 40 distinct genotypes of *Cryptosporidium* have been described, and only 10 species are considered to be valid (Fayer *et al.*, 2000; Thompson, 2002; Plutzer and Karanis, 2009; Dixon, 2014). *Cryptosporidium parvum* is the most important species due to its ability to infect a wide range of mammals including humans (Karanis, 2011). Two genotypes are described within this specie: 1 -H (human stream, transmitted from human to human) and 2 -C (bovine stream, the zoonotic one transmitted between humans and cattle) (Fayer *et al.*, 2004; Xiao and Fayer, 2008). The identification of *Cryptosporidium* genotypes has been made based on either one of following loci; ssrRNA gene, 70 kDa heat shock protein (*hsp 70*) (Xiao *et al.*, 2002) and *Cryptosporidium* oocysts wall protein (*cowp*) (Chalmers *et al.*, 2002).

Table 1: Species and genotypes of *Cryptosporidium* (adapted from Fayer *et al.*, 2000 and Taylor *et al.*, 2007)

Species	Host species	Predilection site	Genotype
<i>C. andersoni</i>	Cattle, mouse	Abomasum	
<i>C. baileyi</i>	Chicken, turkey, duck, cockatiel, quail, ostrich	Small and large intestine, cloaca, bursa of fabricius, nasopharynx, trachea, conjunctiva	
<i>C. meleagridis</i>	Turkey, chicken, duck, parrot	Small intestine	

<i>C. canis</i>	Dog, fox, man	Small intestine			
<i>C. nasorum</i>	Fish	Stomach and small intestine			
<i>C. serpentis</i>	Reptiles	Stomach			
<i>C. wrairi</i>	Guinea Pig	Small intestine			
<i>C. saurophilum</i>	Lizards, snakes	Intestine, cloaca			
<i>C. felis</i>	Cat, cattle, man	Small intestine			
<i>C. muris</i>	Mouse	Small intestine			
<i>C. parvum</i>	Cattle, sheep, goat, horse, deer, man	Small intestine	1	-H	(human genotype)
			2	-C	(bovine genotype)

2.4.4. Life cycle

The life cycle of *Cryptosporidium* spp. is direct (Figure 3) and animals may be infected by one of the following routes; direct contact and ingestion of food or water contaminated by cysts (Fayer *et al.*, 2000; Paraud and Chartier, 2012; Dixon, 2014). The sporulated oocysts (two sporocysts with four sporozoites) are eliminated with faeces in the environment. Once ingested by host, the sporozoites excyst from oocysts in the gastrointestinal tract, (a) invading the microvillous brush border of the enterocytes (b) in mammals or of the proventriculus, intestines and lungs in birds (Taylor *et al.*, 2007). Each sporozoite differentiates into a trophozoites (c) which multiply asexually forming meronts (d) with four to eight merozoites (e). When the host cell matures the meront breaks and merozoites are released to infect new cells (f) (type II or type III meronts). After generations of meronts, gametogony takes place with fusion of macrogamont (g) and microgamont (h) and zygote is produced (i). Sporulation occur within the host. Evidence indicates that two types of oocysts are produced: the thick walled (k) passed in the faeces (j) and the thin walled (l) releasing their sporozoites in the intestine, causing auto-infection (Monis and Thompson, 2003; Taylor *et al.*, 2007; Kanaris, 2011). The cycle is

maintained by resistance of oocysts in the environment for weeks or months (Dixon, 2014). The prepatent and patent period is 3 days and 10-20 days in birds (Taylor *et al.*, 2007) 3-6 and 4-13 days in calves respectively (Fayer *et al.*, 1998).

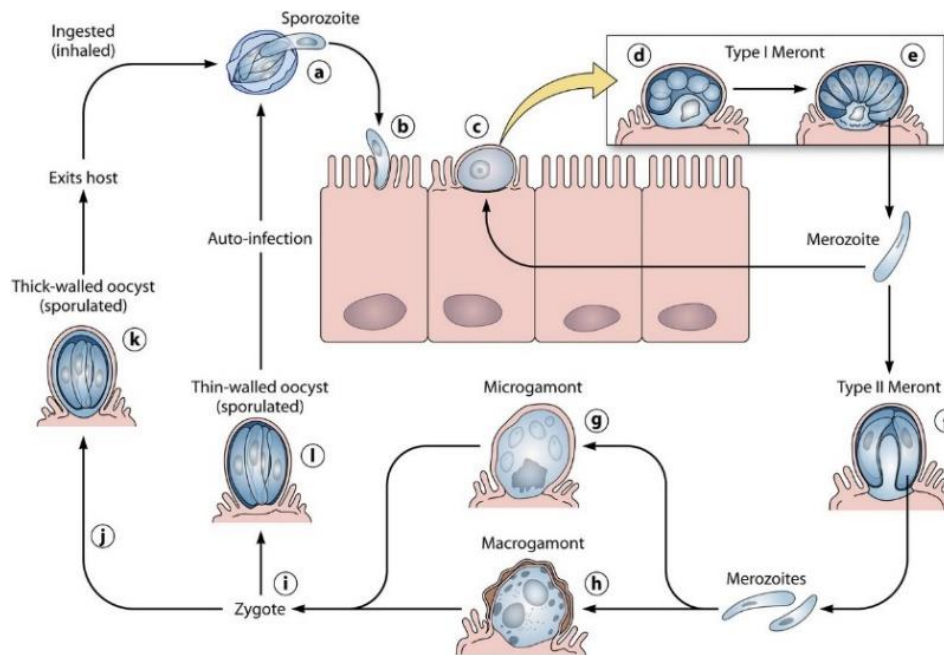


Figure 3: *Cryptosporidium* life cycle (Bouzid *et al.*, 2013)

2.4.5. Pathogenesis and clinical manifestation

Animals infected with *Cryptosporidium* species manifest with diarrhoea as the main clinical symptom. It is believed that this may be derived from one of the following processes; increased secretory flow of water through the outlet of chloride into the intestinal lumen, inhibition of absorption of sodium and the high production of prostaglandins in the intestinal mucosa and increase of γ -interferon level due to increase in the permeability of the intestinal mucosa (Foster and Smith, 2009).

The injury of the intestinal barrier causes reduction in nutrient absorption leading to weight loss and sometimes vomiting, abdominal pain and anorexia may occur (O'Donoghue, 1995; Dawson, 2005; Taylor *et al.*, 2007). Clinical signs appear 3 to 5 days after infection and may have a duration of 8 to 13 days (O'Handley and Olson, 2006).

In many cases the infection is asymptomatic (Dawson, 2005) especially in dogs, cats, horses and pigs (Fayer, 2004). The disease is more prevalent in lambs between the ages of 5-20 days (Paraud and Chartier, 2012) in calves from 1-3 weeks (Wyatt *et al.*, 2010). The mortality may be high in severe cases with malnutrition and concomitant infections (De Graaf

et al., 1999; Fayer, 2004). Respiratory manifestation with sneezing and coughing may occur in infections by *Cryptosporidium baileyi* in birds (Taylor *et al.*, 2007).

2.4.6. Epidemiology

Various factors influence directly the transmission and maintenance of *Cryptosporidium* spp. in susceptible hosts and environment. Since water is one of the main transmission vehicles, the resistance of oocysts to chlorine as well as its small size which makes the treatment of water not effective by classic methods of filtration (Dixon, 2014). The oocysts persist in the environment for long periods (Paraud and Chartier, 2012) and the low dose of oocysts required for new infection after elimination allowing immediate transmission between hosts (Taylor *et al.*, 2007).

Cattle, especially pre-weaned calves are considered to be most important animal species involved in the transmission of cryptosporidiosis (*C. parvum*) in terms of distribution and prevalence; not only because of the large amount of oocysts eliminated to the environment and also that the species is zoonotic (Taylor and Webster, 1998; Fayer, 2004). The risk of zoonotic transmission from dogs and cats is low (Lucio-Forster *et al.*, 2010) with few cases in HIV-positive individuals getting infected by *C. canis* and *C. felis* (Morgan *et al.*, 2000).

The prevalence varies between 2.5% (Castro-Hermida *et al.*, 2005) and 9.5% (Geurden *et al.*, 2008a) in goat kids; 6.2% (Coklin *et al.*, 2009), 12% (Hammes *et al.*, 2006), 47.9% (Castro-Hermida *et al.*, 2002) in calves; 18.1% (Nguyen *et al.*, 2012) and 22.1% in pigs (Johnson *et al.*, 2008).

2.4.7. Treatment and control

There is no treatment with proven efficacy against cryptosporidiosis (Olson *et al.*, 2003). Some studies have reported the efficacy of halofuginone lactate at a dose of 100 mg/kg for 7 days in goat kids as a therapeutic drug (Giadinis *et al.*, 2008) while another study with the same drug was efficacious as a prophylactic (De Waele *et al.*, 2010; Petermann *et al.*, 2014). Nitazoxanide (Schnyder *et al.*, 2009), paromomycin and decoquinatate (Mancassola *et al.*, 1997) are drugs used in the prophylaxis of cryptosporidiosis especially when administered during the first weeks of life, as it reduces the elimination of oocysts to the environment and consequently the exposure of susceptible animals to infection. This treatment should be complemented by the adoption of strict hygiene measures to reduce the availability of oocysts in the environment and contamination among animals (Shahiduzzaman and Dauschies, 2012).

A symptomatic treatment may be instituted in order to prevent diarrhoea, replace fluids and acid-base balance as well as prevent secondary bacterial infections (Taylor *et al.*,

2007). Once the calves are infected they begin to eliminate oocysts starting from the second day of life. Ensuring access to colostrum after birth would be a sustainable alternative for the control, since colostrum has anti-*Cryptosporidium* antibodies (Olson *et al.*, 2003). *Cryptosporidium* oocysts are resistant to most common disinfectants with the exception of 10% formaldehyde, hydrogen peroxide at 3%, ammonia (Taylor *et al.*, 2007) and ozone (Fayer, 2004).

2.4.8. Diagnosis

The routine diagnosis is based on observation of cysts in normal stools or trophozoites in diarrhoeal stools in case of giardiasis (Conboy, 1997) and oocysts in case of cryptosporidiosis (Taylor *et al.*, 2007). This can be done using different techniques such as coprological (flotation concentration techniques, or stained smears followed by observation under light microscope) (Cheesbrough, 1987), immunological (ELISA, Immunofluorescence) or molecular (PCR) (Taylor and Webster, 1998).

Flotation technique is based on using a solution which has a specific gravity (saturated sugar solution, saturated sodium chloride or zinc sulphate solution) that makes eggs or (oo)cysts to float and captured on the surface where they are collected on a cover glass followed by microscope examination (Cheesbrough, 1987; Taylor and Webster, 1998; Ueno and Gonçalves, 1998).

Faecal smears stained by modified Ziehl Neelsen, safranin methylene blue (Cheesbrough, 1987) or auramine phenol (Taylor and Webster, 1998) are successfully implemented in the identification of protozoa (oo)cysts. The disadvantage of these techniques is the low specificity and sensitivity due to the small size of *Cryptosporidium* spp. oocysts that can be confused with algae or yeast (De Waal, 2012) as well as in cases of low elimination of oocysts where concentration techniques prior to staining is necessary (Formol-Ether concentration technique) (Salleh *et al.*, 2014) to improve test sensitivity. Another disadvantage of these techniques is that the results can vary from laboratory to laboratory, often depending on the practice and experience of examiner and false negatives may occur.

The immunofluorescent assay-IFA allows the morphological and immunological identification of *Cryptosporidium* oocysts and *Giardia* cysts. Through this technique the (oo)cysts wall is stained when coupled to a specific monoclonal antibody conjugated with a specific fluorochrome, generally fluorescein isothiocyanate or auramine rhodamine (Direct Immunofluorescence) and a second antibody may also be included (Indirect Immunofluorescence) (Dixon, 2014; Robertson, 2014). A direct immunofluorescence

diagnostic kits that detect both parasites is available (MERIFLUOR *Cryptosporidium/Giardia*, Meridian Bioscience, Inc., Cincinnati, OH) with high sensitivity tested comparing with staining methods (Gómez-Couso *et al.*, 2006; Geurden *et al.*, 2008b). Due to the high sensitivity and specificity (100%) also indicated on this diagnostic kit this, this test has been used in most studies as gold standard (Johnston *et al.*, 2003; Mekaru *et al.*, 2007; Elsafi *et al.*, 2014).

The enzyme-linked immunosorbent assay-ELISA detects faecal antigens having advantage that several samples can be processed at once. By this test positive results are not always indicative of the presence of the parasite, only indicating the contact of susceptible host with parasite, even if it has already been completely eliminated (De Waal, 2012). These techniques are based on the use of monoclonal antibodies for the detection of parasites (Taylor and Webster, 1998) being more sensitive than staining routine tests with the disadvantage of its high cost; and also does not allow the identification of species of parasite involved in the infection.

Polymerase chain reaction (PCR) has been used with great success consisting of amplifying the DNA of the parasite, showing better results compared with techniques already mentioned, due to its high sensitivity and specificity (Taylor and Webster, 1998); enabling diagnosis of samples with low concentrations of parasites, and the identification of the species and genotype involved by combining PCR with restriction fragment length polymorphism (RFLP) and sequencing analysis (Xiao *et al.*, 1999; Widmer *et al.*, 2002; Wyatt *et al.*, 2010). The disadvantage of this technique is the high cost related to the acquisition of reagents and equipment.

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted between February and September 2015 in Magude District, which is the largest producer of cattle in the Maputo province (INE, 2009). The district is located in the northern part of Maputo province between 26° 02'00" south latitudes and between 32°17'00" east longitudes. Is bordered by district of Bilene Macia and Chokwe in the north, by Moamba district in south and by the Republic of South Africa in west (MAE, 2005). The district is divided into five administrative posts; Magude Sede, Mahele, Mapulanguene, Motaze and Panjane (INE, 2009).

The climate is dry subtropical, with an average annual temperature of 22 °C to 24 °C and the average annual rainfall of 630 mm. Two seasons are predominant; hot and heavy rainfall in October to March, cool and dry in April to September (MAE, 2005).

3.2. Sample Collection

The study was conducted in calves, young goats and young dogs (≤ 7 months) of both sexes. Information on the age of animals was obtained from the animal owners. Sampling of cattle was carried out at dip tanks during vaccination campaign and for dogs during the vaccination campaign against rabies. Samples in goats and part of samples in dogs were collected in residences.

To calculate the sample size, the expected prevalence was estimated considering the prevalence already reported in African countries bordering Mozambique such as Tanzania which was 16.3% for *Cryptosporidium* in calves (Mtambo *et al.*, 1997) and 5.6% for *Giardia* in dogs from South Africa (Mukaratirwa and Singh, 2010). An expected prevalence of 20%, precision measured of 95% and confidence interval of 5% were used to calculate the sample size using the following formula described by Thrusfield (1999), where n correspond to the sample size, P_{exp} the expected prevalence, d^2 the precision measured, $n_{adjusted}$ the sample size adjusted and N the population size of Magude District.

$$n = \frac{1.96^2 P_{exp} (1 - P_{exp})}{d^2} \quad n_{adjusted} = (N \times n) / (N + n)$$

Table 2: Sample size calculated based on livestock enrolment (SDAE, 2013)

Animals	Population size	Sample size
Cattle	89 427	245
Goats	7 487	238
Dogs	36 856	244

There was no information regarding the population size of young animals of the district which we assumed would be less if considering the standard distribution of young animals in the animal population. The sample size above referred was determined basing on the total number of animals including adult animals. It was not possible to obtain the referred number of young animals due to the reduced number of offspring in the sampling period, refusal by some farmers to provide their animals for sampling which was related with obscure cultural beliefs and nomadism due to lack of pasture.

The farms sampled were randomly selected according to the presence of animals within the age range referred and according to the availability of animal owners. Samples collected per farm ranged from 1 to 3.

A total of 696 faecal samples were collected (480 from calves, 156 from dogs and 60 from goats) from the rectum of each animal using a latex glove and transferred to tubes with caps which were labeled with individual details of each animal and transported in a cooler box to the Laboratory of Parasitology at Eduardo Mondlane University (UEM) for further processing.

Each sample was equally divided into two aliquots in 2 ml Eppendorf tubes with the same identification from the collection tubes. One aliquot was preserved in absolute ethanol (1:1) for molecular analysis and the other in 10% formalin (1:3) as backup. Remaining samples were stored in a refrigerator unpreserved at 4 °C until processed.

All parasitological analyses were done at the Parasitology Laboratory, Faculty of Veterinary, UEM in Maputo, Mozambique. Molecular analyses were done at the University of KwaZulu-Natal (UKZN), School of Life Sciences, Parasitology section, Westville campus, Durban, South Africa.

3.3. Laboratory analysis of samples

The study consisted of detection and identification of gastrointestinal parasites by flotation technique in NaCl solution (Willis), modified Ziehl-Neelsen (mZN), direct immunofluorescent (DIF), indirect immunofluorescence (IIF) and Polymerase Chain Reaction (PCR) techniques.

Fresh faecal samples were processed for the identification (Willis) and quantification (McMaster) of gastrointestinal parasites as described by Ueno and Gonçalves (1998) (Figure 4). The identification of helminth eggs was performed using the morphological characteristics as described by Taylor *et al.* (2007). To concentrate the (oo)cysts in the faecal samples, the formol-ether technique was used as described by Cheesbrough (1987). The pellet obtained from the concentration was used to prepare thin smears which were stained by the mZN method as described by Cheesbrough (1987) and observed under an optical microscope at 100x magnification using immersion oil for the presence/absence of *Cryptosporidium* oocysts. The remainder of the pellet was transferred to Eppendorf tubes and preserved at -20 °C for further processing for the detection and quantification of *Cryptosporidium* and *Giardia* by DIF and IIF tests.



Figure 4: Aliquots of faecal samples in 2 ml Eppendorf tubes (A), Willis and McMaster test (B)

3.3.1. Direct immunofluorescence

The tests were carried out using a kit (MERIFLUOR® *Cryptosporidium/ Giardia*; Meridian Diagnostic) (Figure 6) according to the manufacturer's specification. Briefly 25µl of concentrated sample was transferred to the well of a treated slide, a negative and positive control were included on each slide. The sample was spread using different transfer loop to avoid contamination, and dried for 30 minutes. One drop of detection and contra-stain reagents were placed on each well, spread and incubated in humid chamber at room temperature for

30 minutes. The slides were washed using the buffer included on the kit and the excess of water removed in paper towel. The observation was made in fluorescence microscope (Olympus, BX 53) using 100x magnification (Figure 6) after adding one drop of mounting reagent and covered with a coverslip. Approximately 50% of samples from each animal species were randomly selected and analyzed by this technique.

3.3.2. Indirect immunofluorescence

25 µl of concentrated faeces by formol-ether method was transferred to immunofluorescence slide (Figure 6D), left dry for approximately five minutes and fixed with absolute methanol. Approximately 50 µl of primary antibody (Anti-*Cryptosporidium parvum* mAb and Anti *Giardia lamblia* pAb, Abnova) diluted in 3% BSA in PBS (1:500) were added to the smear, incubated for 1 hour in a wet chamber and then washed 3 times in PBS Tween-20 (0,05%).

One drop of the secondary antibody coupled to fluorescein (Goat pAb to *Cryptosporidium parvum* oocyst and *Giardia* cysts/FITC, Abcam) diluted in 3% BSA in PBS (1:1000) was added to the smear, incubated in the dark for 30 minutes and washed 3 times to remove excess of fluorescein. To obtain the optimal dilution of the secondary antibody, serial dilutions were made starting from 1:10. A mounting reagent was added in the slide, covered with a coverslip and observed in a fluorescence microscope (100x).

Positive samples by the mZN, DIF and IIF tests were processed for PCR test at the Molecular Laboratory at the School of Life Sciences, University of KwaZulu-Natal (UKZN). The procedure followed in the analysis of samples by different techniques is shown in Figure 5.

The number of *Cryptosporidium* oocysts and *Giardia* cysts per gram of faeces (OPG and CPG) by DIF and IIF test, was determined using the following formula described by Castro-Hermida *et al.* (2007).

$$\text{OPG/CPG} = \frac{\text{Number of oocysts or cysts identified}}{\text{Volume of sample(ml) * Weight of faeces (g)}}$$

The intensity of infection for *Giardia* and *Cryptosporidium* was classified as described by Castro-Hermida *et al.* (2002) and for helminthic infections in livestock as described by Hansen and Perry (1994) and for helminthic infections in dogs (Mukaratirwa and Singh, 2010).

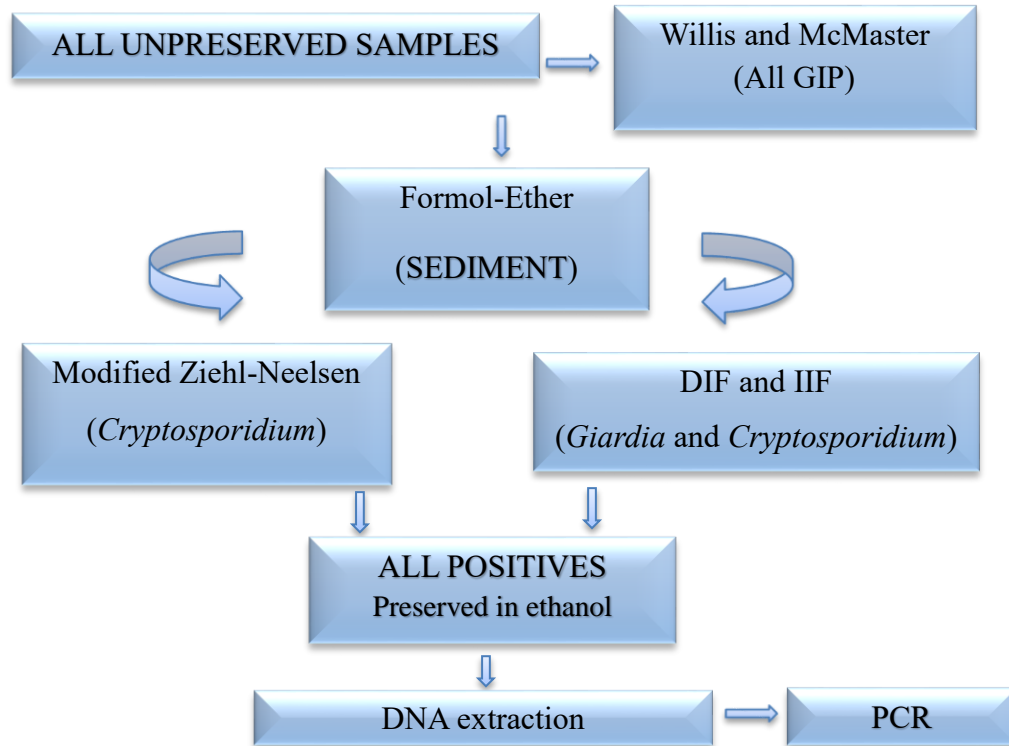


Figure 5: Procedure for selection of diagnostic technique used to detect gastrointestinal parasites in livestock and dogs of Magude District. GIP = Gastrointestinal parasites, DIF = Direct immunofluorescence, IIF = Indirect immunofluorescence test

3.3.3. DNA extraction

The ethanol-preserved samples were pre-cleaned in distilled water using two coupled filters of 100 μm and 20 μm into conical centrifuge 15ml tubes. The solution was centrifuged at 1300 rpm for 5 minutes and the sediment obtained concentrated in sugar solution to allow the parasites to float for 30 minutes as described by Ueno and Gonçalves (1998). The supernatant was recovered using Pasteur pipette and transferred into a 2 ml Eppendorf tube. The sugar was removed by adding distilled water in the tube and centrifuging the sample (1300 rpm for 3 minutes) and the pellet obtained used for extraction of DNA using QIAamp DNA Stool Mini Kit (Qiagen Hilden, Germany) following the manufacture specifications and the eluate stored at $-20\text{ }^{\circ}\text{C}$ until analyzed.

Before extracting DNA, cleaned samples were observed under an optical microscope (40x) to confirm the presence of cyst and oocysts and then the isolated parasites were mechanically disrupted using glass beads and exposed to different freeze-thaw cycles in liquid nitrogen and hot water (Figure 6G) in order to expose the DNA as described by Babaei *et al.* (2011) for *Giardia* spp. and Bialek *et al.* (2002) for *Cryptosporidium* spp.

3.3.4. PCR

A nested PCR was applied according to Xiao *et al.* (1999) and Morgan *et al.* (2001) for *Cryptosporidium* and Lalle *et al.* (2005) for *Giardia* using the primers indicated in Table 3.

Table 3: Primers used for molecular diagnosis of *Cryptosporidium* and *Giardia*

Parasite	Locus	Primer	Size (bp)
<i>Cryptosporidium</i>	SSU rRNA	5'-TTCTAGAGCTAATACATGCG-3' 5'-CCCATTTCTTCGAAACAGGA-3' 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' 5'-AAGGAGTAAGGAACAACCTCCA-3'	830
	HSP70 gene	5'-TA/CTTCATG/CTGTTGGTGTATGGAGAAA-3' 5'-CAACAGTTGGACCATTAGATCC-3' 5'-ATGA/GGA/TGAAGAAGA/TAA/GC/TA/TCAAGC-3' 5'-AGAAG/ACAC/TTTTCTGTGT/GACAAT-3'	1950
<i>Giardia</i>	β -giardin	G7:AAGCCCGACGACCTCACCCGCAGTGC G759:AGGCCGCCCTGGATCTTCGAGACGAC GiarF: GAACGAACGAGATCGAGGTCCG GiarR: CTCGACGAGCTTCGTGTT	753 511
	SSUr RNA	RH11:CATCCGGTCGATCCTGCC RH4: AGTCGAACCCTGATTCTCCGCCC AGG GiarF: GACGCTCTCCCCAAGGAC GiarR: CTGCGTCACGCTGCTCG	292 130

The reaction mixture for the first and secondary PCR consisted of 10 μ l of Taq polymerase master mix, 3 μ l of forward and reverse primers, 4 μ l of sterile water and 5 μ l of DNA to make a final volume of 25 μ l. Visualisation of PCR products were made in agarose gel (1%) and visualized by ethidium bromide.

The protocol used for β -giardin and SSU rRNA consisted of an initial denaturation step at 95 °C for 15 min and final elongation for 7 minutes at 72 °C, denaturation at 95 °C for 30

seconds, annealing at 65 °C for 30 seconds and extension at 72 °C for 60 seconds repeated 45 times. The secondary PCR differed only in the annealing temperature (55 °C for β -giardin and 59 °C for SSU rRNA).

For HSP70 gene an initial denaturation of 96 °C for 2 minutes and final extension at 72 °C for 4 minutes followed by 40 cycles of annealing temperature at 96 °C for 30 seconds, 58 °C for primary and 55 °C for secondary annealing for 30 seconds and extension at 72 °C for 45 seconds. To amplify the SSU rRNA gene, the following conditions were applied, 94 °C for 45 seconds, 55 °C for 45 seconds and 72 °C for 1 minute, using an initial denaturation of 94 °C for 3 minutes and a final extension of 72 °C for 7 minutes. The same conditions were used for the secondary PCR.



Figure 6: Primary, second antibodies (C) and slides used for Indirect Immunofluorescence test (D), Immunofluorescence microscope (Olympus BX53) (E), Kit used for Direct Immunofluorescence test (MERIFLUOR® *Cryptosporidium/ Giardia*) (F) and disruption of oo(cysts) in freeze-thaw cycles (G)

3.3.5. Questionnaire

A questionnaire was designed for dog owners and livestock farmers to collect information regarding animal husbandry, housing conditions, drinking water sources, feeding, treatment against parasitic infections and use of faeces in agricultural practices. The collected information was used to determine the risk factors related to transmission of *Giardia* and *Cryptosporidium*.

3.3.6. Data analysis

A sample was considered positive if at least one cyst/oocyst of *Giardia/Cryptosporidium* was identified. The prevalence (%) was calculated as the number of positive samples divided by the total number of collected samples multiplied by 100 and the results were expressed with a 95% Confidence Intervals (95% CI) (Thrusfield, 1999). To analyse differences in the prevalence of *Giardia* and *Cryptosporidium* among localities of Magude District, a general linear model multivariate was applied. Data from the questionnaire were introduced in Excel program to determine risk factors associated with *Giardia* and *Cryptosporidium* transmission using Chi Square or Fisher exact test and relative risk. All data were exported to SPSS program version 20.0 and p -value < 0.05 was considered to be statistically significant. Medicalc software was used to calculate the sensitivity and specificity of mZN and IIF with the DIF test used as gold standard.

4. RESULTS

The following results are referent to the analysis of 480 faecal samples from calves, 60 from goats and 156 from dogs from the familiar sector of Magude District. Crossbred animals of both sexes and aged between 0 and 7 months were involved. The number of questionnaires was the same as the animals and all the questions were answered.

4.1. Prevalence of gastro-intestinal protozoa

Giardia cysts were detected in calves, young goats and dogs and *Cryptosporidium* oocysts in calves and dogs by the different methods used, and variations in the prevalence were observed according to animal species involved and the diagnostic test applied (Figure 7). A higher prevalence of *Giardia* was observed in dogs and calves by IIF [8.3% (CI: 8.0 - 8.5) and 8.1% (CI: 7.9 - 8.3)] than by DIF test [5.7% (CI: 5.4 - 5.9) and 6.0% (CI: 5.8 - 6.2) respectively]. Trophozoites of *Giardia* were detected in one sample from a dog by Willis technique [0.6% (CI: 0.52 - 0.67)]. Since the secondary antibody for IIF was derived from goats (goat pAb *Cryptosporidium* and *Giardia* oocyst - FITC), this method was not applied in goats. However, the prevalence of *Giardia* in goats by DIF test was higher than in dogs and calves [13.3% (CI: 12.5 - 14.0)]. All positive samples by mZN, DIF and IIF were negative to PCR test.

In calves and dogs, the prevalence of *Cryptosporidium* was also high by IIF [4.7% (CI: 4.5 – 4.8) and 6.4% (CI: 6.1 – 6.6)] followed by mZN test [3.8% (CI: 3.6 – 3.9) and 0.6% (CI: 0.52 – 0.67)] and finally by DIF test [0.4% (CI: 0.35 – 0.44) and 0.64% (CI: 0.62 – 0.65)] respectively. The parasite was not diagnosed in goats by any of the mentioned tests and by Willis technique in all animal species. In addition to the already mentioned protozoal parasites, cysts of *Sarcocystis* spp. [3.8% (CI: 3.6 – 3.9)] and oocysts of *Isospora* spp. [2.6% (CI: 2.4 – 2.7)] were detected by Willis technique in faecal samples of dogs. All samples collected in calves and goats had normal consistency and in dogs, 3 from 156 samples were diarrhoeal (1.9%) and from these only one was positive for trophozoites (0.6%).

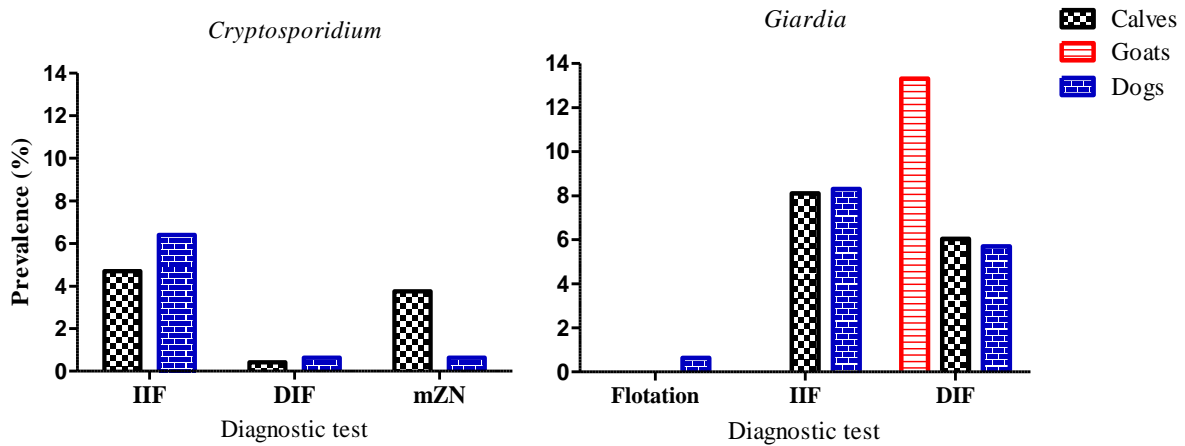


Figure 7: Prevalence of *Giardia* species and *Cryptosporidium* species in calves (n=480), goats (n=60) and dogs (n=156)

A total of 20 randomly selected fields were microscopically observed in all processed samples by DIF and IIF tests and the number of *Giardia* cysts and *Cryptosporidium* oocysts (Figure 8) had the same distribution ranging from 0 to 2 oo(cysts). The same was observed by mZN test where on average 2 oocyst of *Cryptosporidium* were observed (Figure 9) per sample. The number of *Giardia* cysts and *Cryptosporidium* oocysts per gram of faeces (CPG and OPG) in dogs ranged between 20 and 80, in calves the OPG ranged between 40 and 160, the CPG between 20 and 40, and the same CPG was recorded in goats.

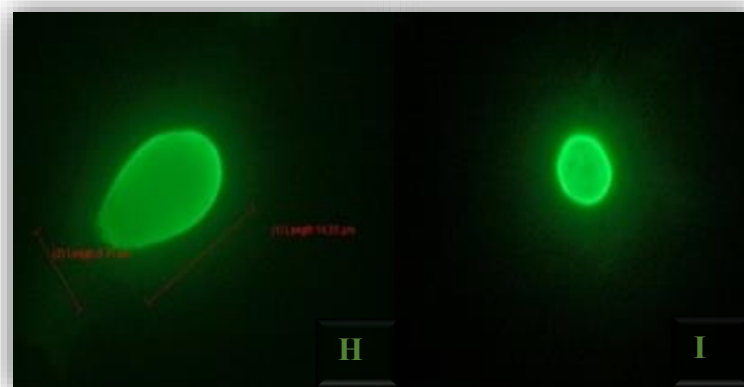


Figure 8: *Giardia* spp. cysts (H) and *Cryptosporidium* spp. oocyst (I) detected by Direct Immunofluorescence test (100x amplification) in faecal sample of dog

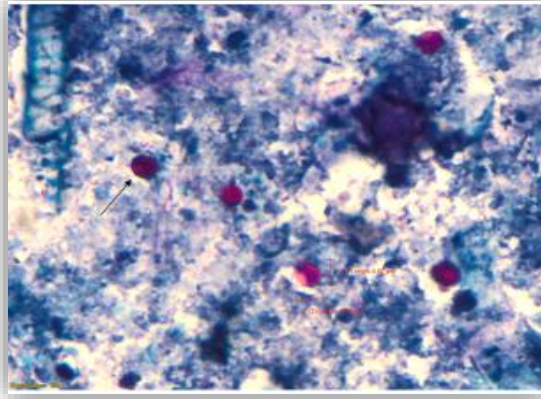


Figure 9: Oocysts of *Cryptosporidium* spp. stained by modified Ziehl Neelsen method in faecal smears of calves (100x amplification), spherical structures red in colour

Comparing the prevalence of these parasites among different localities of Magude District showed that the highest number of infected calves and dogs by *Giardia* spp. were registered in the locality of Magude Sede (5.2% and 7.1% respectively) and the lowest in the locality of Mahele (0.2% and 0.6%). The locality of Mahele differed significantly from the locality of Magude Sede in the occurrence of *Giardia* spp. in calves. The prevalence rate of *Giardia* spp. in goats from Motaze and Magude Sede was the same (3.3%) and in other localities no positive samples were detected.

The prevalence of *Cryptosporidium* spp. was high in calves from Motaze and Magude Sede (2.0%) and in dogs of Magude Sede (5.8%) when compared with other localities, however, the differences observed were not statistically significant ($p > 0.05$).

When all positives obtained by different diagnostic tests are combined, the prevalence of *Giardia* spp. was higher than *Cryptosporidium* spp. In all animal species as shown in the Table 4.

Table 4: Prevalence (%) of *Giardia* and *Cryptosporidium* in different localities of Magude District, Mozambique

Locality	Calves (480)		Goats (60)		Dogs (156)	
	Prevalence (%)					
	<i>Giardia</i>	<i>Cryptosporidium</i>	<i>Giardia</i>	<i>Giardia</i>	<i>Cryptosporidium</i>	
Motaze	4 (0.8)	10 (2.0)	2 (3.3)	0	0	
Mahele	1 (0.2)*	4 (0.8)	0	1 (0.6)	0	
Mapulanguene	6 (1.3)	3 (0.6)	0	2 (1.3)	0	
Panjane	6 (1.3)	5 (1.0)	0	2 (1.3)	1 (0.6)	
Magude Sede	25 (5.2)	10 (2.0)	2 (3.3)	11 (7.05)	9 (5.8)	
Total	42 (8.8)	32 (6.6)	4 (6.6)	16 (10.3)	10 (6.4)	

**p*-value based on general linear model multivariate (*p* = 0.001)

4.2. Evaluation of sensitivity and specificity

The sensitivity and specificity of IIF, Willis and mZN are presented in the Table 5. Eight samples were positive to *Giardia* spp. in dogs by DIF and IIF test (true positives) and seven samples were positive by IIF test but negative by DIF (false positive). For *Cryptosporidium* spp. one sample was positive by DIF and mZN tests (one true positive), and nine samples were positive only by IIF (false positive). In calves one sample was positive to *Cryptosporidium* spp. by mZN, IIF, DIF and 28 samples were positive for *Giardia* spp. by DIF and IIF (true positives).

The mZN test showed high sensitivity (100%) and specificity (96.20% and 100%) to detect *Cryptosporidium* infections in calves and dogs respectively. The IIF test showed high sensitivity and specificity to both parasites, ranging the sensitivity between 88.89% and 100%, and specificity between 95.38% and 98.51% for *Giardia* spp.; sensitivity of 100% and specificity of 93.15% and 93.9% for *Cryptosporidium* spp.

The Willis test did not prove to be a sensitive test for detection of *Giardia* cysts and *Cryptosporidium* oocysts, and one false positive to *Giardia* spp. was detected in dogs. The specificity was from 98.46% and 100% for *Giardia* spp. and 100% for *Cryptosporidium* spp.

4.3. Risk factors for *Giardia* and *Cryptosporidium* transmission

Water source for animals was identified as a risk factor involved in the transmission of *Giardia* spp. and *Cryptosporidium* spp. in calves and dogs (*p* < 0.05) as shown in the Tables 6 and 7. Most calves had the river as the main source of water (27 from 32 positives to

Cryptosporidium spp. and 39 from 42 positives to *Giardia* spp.). The results also showed that calves were approximately 4 times more likely to contract *Cryptosporidium* (OR: 11.6; 95% CI: 3.48-39.27) than *Giardia* (OR: 3.66, 95% CI: 0.95-14.13) due to contact with river water. The same was observed in dogs where the animals were 2 times more likely to be infected by *Cryptosporidium* (OR: 5.23; 95% CI: 1.29-21.18) than by *Giardia* (OR: 2.52; 95% CI: 0.99-6.40). From 42 calves positives to *Giardia* spp., 39 never had treatment against parasitic infections, constituting also a risk factor for *Giardia* transmission ($p < 0.05$). In goats no risk factor was related to the transmission of *Giardia* ($p > 0.05$).

Table 5: Sensitivity and specificity (95% CI) of diagnostic tests used in the detection of *Cryptosporidium* oocysts and *Giardia* cysts in dogs and calves

Test	<i>Cryptosporidium</i> spp. (95% CI)				<i>Giardia</i> spp. (95% CI)			
	Dogs		Calves		Dogs		Calves	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Willis	0%	100%	0%	100%	0%	98.46%	0%	100%
	(0-97.5%)	(95.1-100%)	(0-84.2%)	(93.4-100%)	(0-33.63%)	(91.7-99.96%)	(0-11.94%)	(98.17-100%)
mZN	100%	100%	100%	96.20%	–	–	–	–
	(2.5-100%)	(95.1-100%)	(15.8-100%)	(93.2-98.5%)				
IIF test	100%	93.15%	100%	93.9%	88.89%	95.38%	100%	98.51%
	(2.5-100%)	(84.7-97.74%)	(15.8-100%)	(89.9-96.6%)	(51.7-99.7%)	(87.1-99.0%)	(88.1-100%)	(95.7-99.69%)

Table 6: Risk factors associated with transmission of *Giardia* and *Cryptosporidium* infections in calves and goats in Magude District, Mozambique

FACTOR	Calves								Goats			
	<i>Cryptosporidium</i> spp.				<i>Giardia</i> spp.				<i>Giardia</i> spp.			
	<i>np</i>	OR	CI 95%	<i>p</i> -value	<i>np</i>	OR	CI 95%	<i>p</i> -value	<i>np</i>	OR	CI 95%	<i>p</i> -value
Diarrhoea												
Yes	0	1.01	1.00-1.02	0.548	0	1.01	1.00-1.02	0.48	0	1.07	0.99-1.15	0.59
No	32				42				4			
Source of water												
Treated	5	11.6	3.48-39.27	0.00*	3	3.66	0.95-14.13	0.04*	0	1.39	1.16-1.67	0.21
River	27				39				4			
Feeding												
Pasture	31	0.42	0.04-3.60	0.41	41	0.56	0.06-4.84	0.60	4	1.04	0.98-1.11	0.67
Ration	1				1				0			

Cleaning/removal of faeces												
Yes	9	0.28	0.13-0.60	0.76	14	0.71	0.37-1.37	0.53	1	2.73	0.26-28.41	0.38
No	23				28				3			
Deworming												
Yes	3	0.41	0.12-1.40	0.14	3	0.30	0.09-0.99	0.03*	1	3.50	0.29-41.9	0.29
No	29				39				3			
Faeces in agriculture												
Yes	11				18				1			
No	21	1.14	0.53-2.43	0.73	24	1.70	0.89-3.24	0.10	3	1.54	0.14-16.80	0.72

Table 7: Risk factors associated with transmission of *Cryptosporidium* and *Giardia* transmission in dogs in Magude District, Mozambique

FACTOR	<i>Cryptosporidium</i> spp.				<i>Giardia</i> spp.			
	<i>np</i>	OR	CI 95%	<i>p</i> -value	<i>np</i>	OR	CI 95%	<i>p</i> -value
Diarrhoea								
Yes	0	1.02	0.99-1.04	0.64	1	3.32	0.28-38.37	0.30
No	10				20			
Source of water								
Treated	3	5.23	1.29-21.18	0.01*	1	2.52	0.99-6.40	0.04*
River	7				11			
Feeding								
Food scraps	6	1.07	0.29-3.97	0.91	11	0.75	0.30-1.90	0.55
Seeking food	4				10			
Hygienic conditions								
Yes	8	3.12	0.64-15.21	0.14	6	2.0	0.73-5.46	0.17
No	2				15			
Deworming								
Yes	1	0.97	0.11-8.19	0.97	0	1.13	1.06-1.20	0.096
No	9				21			

4.4. Helminthic parasites in dogs

Five helminth parasites (*Ancylostoma* spp., *Toxocara canis*, *Trichuris vulpis*, *Spirocerca lupi* and Taeniidae) were identified in faecal samples of dogs by Willis technique (Figure 12). High prevalence was recorded for *Ancylostoma* spp. [60.3% (CI: 59.8 – 60.7)] followed by *Toxocara canis* [5.8% (CI: 5.6 – 5.9)], *Taeniidae* [1.9% (CI: 1.8 – 2.0)], *Trichuris vulpis* [1.3% (CI: 1.2 – 1.4)] and finally *Spirocerca lupi* [0.6% (CI: 0.5 – 0.7)].

The intensity of infections by zoonotic helminths such as *Ancylostoma* spp. and *Toxocara canis* is shown in Table 8. From a total of 66.1% of infected animals, 60.3% were positive for *Ancylostoma* spp. and only 5.8% for *Toxocara canis*. An intensity range of between

50-500 EPG for *Ancylostoma* spp. was recorded in 35.9% animals and only 1.3% had higher intensity of infection (>5000 EPG). For *Toxocara canis*, infections with intensity of between 50 and 500 EPG were mostly recorded (3.2%).

Table 8: Intensity of infection of gastrointestinal helminths in dogs in Magude District, Mozambique

Parasite	Range of EPG <i>np</i> (%)					Overall prevalence
	50-500	501-1000	1001-2000	2001-5000	>5000	
<i>Ancylostoma</i> spp	56(35.9)	13(8.3)	13(8.3)	10(6.4)	2(1.3)	94(60.3)
<i>Toxocara canis</i>	5(3.2)	1(0.6)	3(1.9)	0	0	9(5.8)
Total	61(39.1)	14(8.9)	16(10.2)	10(6.4)	2(1.3)	103(66.1)

Mixed infections with two or three different helminths or protozoa were observed and *Ancylostoma* spp. was involved in all associations as shown in Table 9. *Ancylostoma* spp. and *Toxocara canis* (4.5%) co-infection was the most observed.

Table 9: Distribution of mixed infections by helminths and protozoa in dogs in Magude District, Mozambique

Parasites	Occurrence (%)
<i>Ancylostoma</i> spp. + <i>Toxocara canis</i>	4.5
<i>Ancylostoma</i> spp.+ <i>Giardia</i> spp.	3.2
<i>Ancylostoma</i> spp. + <i>Cryptosporidium</i> spp. + <i>Giardia</i> spp.	2.6
<i>Ancylostoma</i> spp. + <i>Cryptosporidium</i> spp.	1.9
<i>Ancylostoma</i> spp. + Taeniidae + <i>Cryptosporidium</i> spp.	0.6
<i>Ancylostoma</i> spp. + <i>Sarcocystis</i> spp. + <i>Cryptosporidium</i> spp.	0.6
<i>Ancylostoma</i> spp. + <i>Toxocara canis</i> + <i>Sarcocystis</i> spp.	0.6

4.5. Helminthic parasites and coccidia in calves and goats

In both animal species, eggs of Strongylid, *Moniezia* spp. and oocysts of *Eimeria* spp. were observed by Willis test (Figure 12). In calves the prevalence of Strongylid was high [50.8% (CI: 50.2 – 51.3)] followed by *Eimeria* spp. [17.5% (CI: 17.1 – 17.8)] and *Moniezia* spp. with the lower prevalence [3.3% (CI: 3.1 – 3.4) and 11.6% (CI: 10.8 – 12.3)] in calves and goats respectively). In contrast, the prevalence of *Eimeria* spp. in goats was high [41.6% (CI: 40.4 – 42.7)] followed by Strongylid [31.6% (CI: 30.5 – 32.6)] as shown in Figure 10.

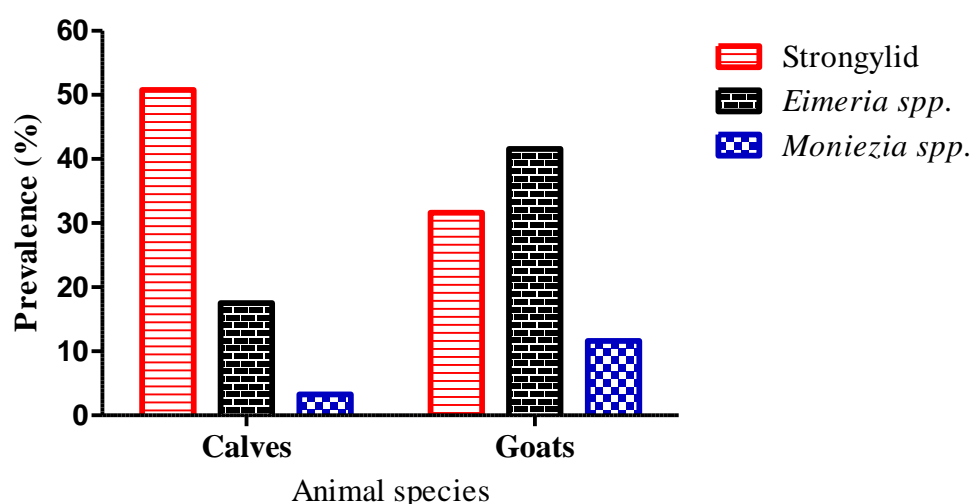
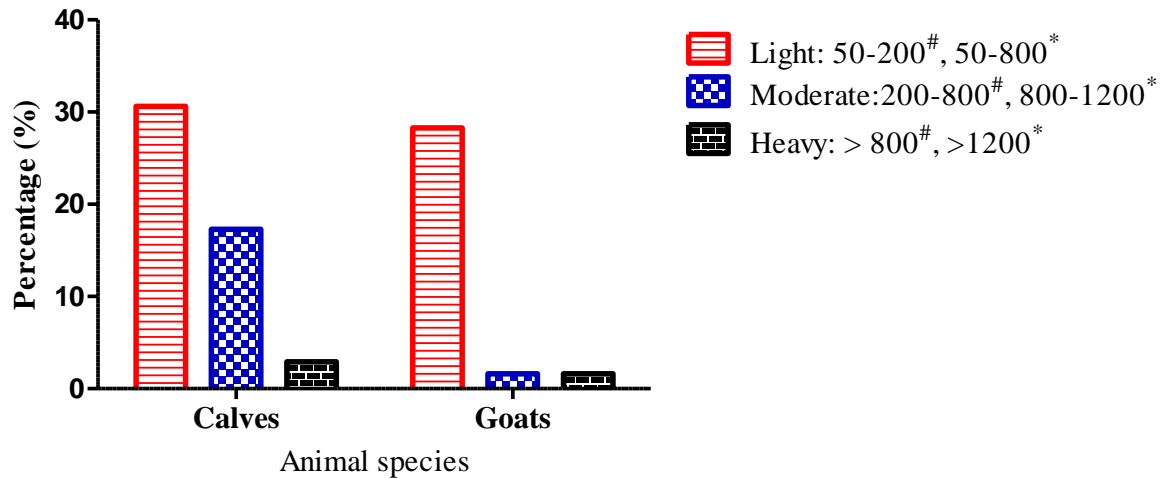


Figure 10: Prevalence of helminths and *Eimeria* spp. by Willis technique in calves and goats in Magude District, Mozambique

Light intensity of infection by strongylids was mostly recorded in both animal species as shown in Figure 11. The intensity of infection by *Eimeria* spp. in Goats ranged between 50 and 20600 OPG, in calves between 50 and 5000 OPG. Low intensities of infection were mostly recorded for both animal species, in calves 14.3% animals had OPG between 50 and 500 OPG, 2.8% with 500 and 4000 OPG and 0.4% with 4000 and 5000 OPG. In goats OPG between 50 and 8000 were recorded in 35% of positive animals, between 8050 and 10000 OPG in 5% and 10050 and 20600 OPG in 1.6% animals.



EPG from calves (#) and goats (*)

Figure 11: Intensity of infection of strongylids in calves and goats by Willis technique



Figure 12: (K) *Toxocara canis*, (L) *Ancylostoma* spp., (M) *Spirocerca lupi*, (N) *Sarcocystis* spp., (O) *Trichuris vulpis*, (P) *Moniezia* spp., (Q) Strongylid egg and (R) oocyst of *Eimeria* spp. detected by Willis technique in faecal samples of dogs and livestock

5. DISCUSSION

The present study focussed on gastrointestinal parasites of livestock and dogs as detected by coprological and immunological tests with special attention to *Giardia* spp. and *Cryptosporidium* spp. This is the first study in Mozambique in a rural community set-up. Several factors may be related to the lack of information about giardiasis and cryptosporidiosis in animals particularly in the south of Mozambique. This may be due to lack of prioritization of livestock diseases as well as lack of sensitive tests in the list of routine diagnostic techniques in laboratories (Bialek *et al.*, 2002) and also the attribution of cases of diarrhoea in young animals to bacterial or viral infections (Holland, 1990).

Due to the lack of demographic and statistical data relating to the distribution and population size of young dogs and goats, it was not possible to estimate the sample size to be collected by locality, so the samples were only taken from residences for us identified with collaboration of the farmers. Most dogs taken to dipping tanks for vaccination were above the age required for this study so most samples were taken house by house. The same obstacle we had with respect to sampling from calves, we had access only to the animals taken to dipping tanks and most farmers did not allow us to have access to new born left at home due to cultural beliefs. This may have contributed to a non-uniform and representative sampling of the population of dogs, goats and calves of Magude District.

The results obtained confirmed the existence of *Cryptosporidium* spp. and *Giardia* spp. in domestic animals in Magude District and the potential risk factors involved in the transmission. Depending on the diagnostic tests applied, there was a wide variation in the prevalence of these parasites.

The prevalence of 3.75% for *Cryptosporidium* spp. found in calves by mZN in the present study is within the range found by Mtambo *et al.* (1997) in Tanzania in calves with less than 8 months. Geurden *et al.* (2006) and Goma *et al.* (2007) in Zambia reported prevalence of 6.3% in calves under traditional husbandry and 4.3% in goat kids respectively using ELISA test which is slightly above the prevalence found in this study.

Results from the immunofluorescence tests in this study showed a lower prevalence of *Cryptosporidium* spp. and *Giardia* spp. than results reported by Hamnes *et al.* (2006) and this may be due to the study of a lower age group (calves between 3 and 183 days) compared to the present study where animals up to 7 months were included. This fact is supported by Mtambo *et al.* (1997) and De Waal (2012) where the prevalence of *Cryptosporidium* spp. varied according to animal age group, being high in new born animals than young animals. In case of *Giardia* infection in dogs, the prevalence found using IIF and DIF test (8.3% and 5.7%) were

below the prevalence reported by Traub *et al.* (2009). In general, the prevalence of *Cryptosporidium* spp. detected by IIF test was higher compared to mZN test, similar results were reported by Mtambo *et al.* (1997).

Cryptosporidium spp. was not detected in goats using all the tests applied in this study. This might have been due to the small sample size obtained in goats due to low birth rate at the time of sampling.

Different studies reported in goats between the age of 5 and 12 months (Castro-Hermida *et al.*, 2005), until 3 months (Goma *et al.*, 2007) between 1 day and 10 weeks (Geurden *et al.*, 2008a) and until one month (Delafosse *et al.*, 2003) showed prevalence of *Cryptosporidium* spp. of 2.5%, 4.8%, 9.5% and 16.2% respectively. These studies show that the prevalence of *Cryptosporidium* spp. was higher in young animals. This may also have been an underestimation of the age of goats involved in the study or the absence of *Cryptosporidium* infections in goats in Magude District.

Comparing the prevalence detected by selecting positive animals from all applied tests, *Giardia* spp. was the most prevalent parasite in all animal species and this finding is similar to study made by Mekarū *et al.* (2007) involving both parasites.

Diarrhoea is a common clinical sign in animals infected by *Giardia* spp. and *Cryptosporidium* spp. (O'Donoghue, 1995; Dawson, 2005). In this study, the presence of diarrhoea was observed in one out of 156 dogs tested for *Giardia*, however no association was observed between case of diarrhoea and the presence of parasite ($p > 0.05$). In this diarrhoeal sample, trophozoites of *Giardia* were observed agreeing with Geurden *et al.* (2010) who reported the frequent presence of trophozoites in diarrhoeal faeces compared to normal faeces.

The presence of these parasites with low intensity of infection suggests the low level of environmental contamination by these parasites in the Magude District. According to Ramirez *et al.* (2004) the amount of excreted oocysts can be a basis for the evaluation of environmental contamination level. Usually the peak of excretion of *Cryptosporidium* oocysts in cattle occurs in the age of 14 days (Olson *et al.*, 2004) and this peak may coincide with the peak of animals with diarrhoea (between 8 to 14 days) as demonstrated by Causapé *et al.* (2002). For *Giardia* infections the peak of excretion occurs between 2 and 4 weeks (Geurden *et al.*, 2010). Perhaps most of the animals involved in this study were out of the age on which the peak of excretion occurs.

Besides the factors already mentioned, others may be associated with the absence of clinical signs in positive animals and low intensity of infection: (i) the development of an immunological response with the advancing age of animals and intermittent excretion of cysts

in *Giardia* infections (Huber *et al.*, 2005) which can result in the apparent low intensity of infection if the study involves only one sampling and (ii) the virulence of the strain involved (Adam, 2014).

The low incidence of animals with diarrhoea may suggest the low pathogenic significance of these parasitic infections in dogs and calves of Magude District. Nevertheless the zoonotic potential of these parasites should be taken in consideration, mainly in case of *Cryptosporidium* infections which is an opportunist in HIV infections (Morgan *et al.* 2000). Clavero *et al.* (1999) isolated this parasite in HIV infected humans in Mozambique.

The evaluation of sensitivity and specificity for Willis, mZN and IIF techniques compared to DIF test showed a high sensitivity (100%) and specificity (96% to 100%) for mZN test in the detection of *Cryptosporidium* infections. The results indicate that the mZN technique is highly reliable in the diagnosis of *Cryptosporidium* spp. in faecal samples. Studies conducted by Zagloul *et al.* (2013) and Quílez *et al.* (1996) in the diagnosis of *Cryptosporidium* spp. by mZN test indicated low sensitivities (73.3% and 79.3% respectively) and specificity approximating to the present study (95% and 100% respectively). The high sensitivity of mZN test to detect *Cryptosporidium* spp. in this study can be attributed to the concentration of oocysts in faecal samples using the formalin-ether technique prior to analysis by subsequent tests. Salleh *et al.* (2014) demonstrated that the sensitivity of mZN can be improved by the application of concentration techniques.

In general, the sensitivity of IIF and mZN tests in detection of *Cryptosporidium* spp. was similar (100%) in this study, these results were also similar to findings by Rimhanen-Finne *et al.* (2007). Despite similarity of results, the choice of diagnostic technique often depends on the availability of resources, time and the objective to be reached (diagnosis of specific parasite or multiple parasites) (Chalmers, 2014). The IIF technique is easy to read due to the incidence of the fluorescent light in oo(cysts), low risk of false positives due to the use of specific antibodies against antigens produced by the parasite and is indicated especially in cases of low intensity of infections (Robertson, 2014). The disadvantage of mZN staining is that the oocysts may be easily confused with faecal debris that take up the stains (Casemore *et al.*, 1985). The efficiency of IIF compared to mZN was reported by Ortega-Mora *et al.* (1999) where concentrated faecal samples of ewes were negative when analysed by mZN and positive by IIF.

Based on the questionnaires responses from animal owners, two factors showed an association with the occurrence of *Giardia* spp. and *Cryptosporidium* spp. in animals, the

source of water (mostly the river which was shared between cattle and humans) and lack of treatment against *Giardia* infections in calves ($p < 0.05$).

Cattle grazing close to rivers may serve as source of contamination of this water by oo(cysts) of *Giardia* spp. and *Cryptosporidium* spp. The cysts/oocysts of these parasites can persist in water for a long period or in the surrounding environment if hot and humid conditions prevail (O'Handley and Olson, 2006; Tangtrongsup and Scorza, 2010). Once in the water, the dispersion of the parasitic forms can be quickly quickened if the water is used for irrigation and other different domestic purposes or by using of animal faeces as fertilizer in agriculture (Slifko *et al.*, 2000; Karanis, 2011).

Since the source of water for livestock and the humans in Magude District was the same, together with the use of this water without any treatment (MAE, 2005) the risk of transmission between animals and humans may be higher (Geurden *et al.*, 2010; De Waal, 2012). Infection of susceptible hosts occurs immediately after the consumption of contaminated water or food (Thompson, 2004; Volotão *et al.*, 2007) as the parasitic forms are already infective when eliminated to the environment with faeces (O'Handley and Olson, 2006).

The lack of anti-protozoal treatment in calves was another risk factor ($p < 0.05$) identified in the maintenance of *Giardia* cycle. Once contaminated if no effective treatment is instituted the elimination of parasitic forms to the environment will be continued, and few cysts would be sufficient to install new infection in exposed animals (Adam, 2014). In the presence of low intensity of infection together with lack of clinical signs as observed in the present study, the decision of treatment must be carefully evaluated in order to avoid the development of resistance and unnecessary costs, with the exception of cases in which animals are permanently in contact with immunosuppressed individuals (Tysnes *et al.*, 2014).

In terms of public health, there is little evidence of the involvement of cattle in zoonotic transmission cycle of *Giardia* spp. compared to dogs, and cattle are mostly linked with the transmission of *Cryptosporidium* spp. (Olson *et al.*, 2004; O'Handley and Olson, 2006). From the present study it is not possible to determine the zoonotic potential of these parasites in Magude District until molecular characterization of the isolates have been done.

Negative results for PCR in the present study were possibly related to low concentration of DNA in the samples diagnosed as positive by mZN, DIF and IIF due to low number of cysts and oocysts in the positive faecal samples (less than 180 OPG and 80 CPG). Similar results have been reported in a study made by Castro-Hermida *et al.* (2007) where they could not

amplify the DNA of *Giardia* and *Cryptosporidium* in samples with less than 800 OPG and CPG. According to Chalmers (2014) the PCR test is sensitive in samples with ≥ 200 oocysts of *Cryptosporidium* per gram of faeces and the number of OPG quantified in the present study was below of the limited referred.

The predominance of low intensity of infection by Strongylid in calves and goats of the present study may be related to the low incidence of rain in the period of sample collection. Sissay *et al.* (2007) observed low EPG counts in sheep during the season with low rainfall compared to wet season. The absence of clinical signs related to coccidiosis or GI helminthic infections may be related to the low pathogenicity of species of *Eimeria*, and Strongilid associated with the low intensity infection (Urquhart *et al.*, 1998), that could only be confirmed if the identification at the level of species had been made.

Ancylostoma spp. (60.25%) and *Toxocara canis* (5.76%) diagnosed in dogs of Magude District pose a risk of infecting people who have contact with dogs due to its zoonotic ability (Taylor *et al.* 2007). The occurrence of the same parasites had already been reported by Cruz and Siva (1971) in Mozambique and Santos *et al.* (2013) in the Veterinary Hospital School in Maputo-Mozambique. In South Africa the prevalence of *Ancylostoma* spp. (53.8%) and *Toxocara canis* (7.9%) reported by Mukaratirwa and Singh (2010) is comparable to the findings of present study, in contrast Minnaar *et al.* (2002) also in South Africa reported high prevalence of *Toxocara canis* (21%).

The epidemiology of *Ancylostoma* spp. and *Toxocara* spp. in dogs is mainly associated with the high prolificacy of females of this parasite and with transmammary infection by which larvae of this parasites are transmitted to the offspring (Urquhart *et al.*, 1998; Taylor *et al.*, 2007). The lower prevalence of *Toxocara canis* comparing with *Ancylostoma* spp. can be justified by the possible presence of animals with larvae in somatic migration in which, instead of the larvae mature and produce eggs, they may have migrated to different tissues where they remained in latency, reducing the rate of excretion of eggs by faeces (Taylor *et al.*, 2007).

Due to the existence of many species of wildlife in Magude District, hunting is one of the activities practiced by the population (MAE, 2005), in this context and according to the information given by dog owners in the questionnaires, the dogs are mainly acquired to aid in hunting and shepherd livestock. In rural areas the level of environmental contamination by eggs or larvae of *Toxocara canis* and *Ancylostoma* spp. tend to be higher due to low sanitary conditions, lack of veterinary assistance and uncontrolled growth of canine population,

increasing the contact between animals from different farms and dispersion of contaminated faeces in the environment (Traub *et al.*, 2005).

In the approach of zoonotic parasites, the circulation of wild animals near the residential areas should be assessed since they can act as reservoirs and asymptomatic carriers (Thompson, 2013). Special attention have been given to Taeniidae parasite of genus *Echinococcus* which has a sylvatic and domestic cycle (Romig *et al.*, 2015). For our study there is a gap related to the genus of Taeniidae diagnosed in dogs because of the inability to differentiate eggs of parasite from this family by light microscope due to their similar morphology (Urquhart *et al.* 1998). Despite the fact that these helminths constitute a health hazard for humans, the impact on animal welfare and health should not be ignored. Veterinarians and public health officials have the responsibility of ensuring the effective control with concrete actions in the communities giving information about these parasites and in the level of different farming systems in order to limit the environmental contamination and new infections.

5.1. CONCLUSION

Giardia and *Cryptosporidium* are prevalent in livestock and dogs of Magude District but there was no evidence of their involvement in zoonotic transmission and in cases of diarrhoea in animals. However special attention should be given to the water source in order to limit the contamination by this parasites since it has been identified as the main risk factor for the transmission of these parasites. Low intensities of infection were mainly recorded for helminthic parasites of dogs (*Toxocara canis* and *Ancylostoma* spp.), cattle and goats (Strongylid and *Eimeria* spp.), even so, the risk of transmission of *Ancylostoma* spp. and *Toxocara canis* to humans should be considered.

5.2. RECOMMENDATIONS

The following future studies, are recommended:

- Further studies be done for an extended period of time to improve the sample size and compare the rate of oo(cyst) shedding between different age groups of animals
- The molecular test should be done from positive samples with high number of oocysts/cysts of *Cryptosporidium* and *Giardia*
- Serial sampling in the same animal group should be done to determine the kinetic of excretion of oo(cysts)
- Collection of samples from the sources of water to detect the presence of *Cryptosporidium* oocysts and *Giardia* cysts for molecular analysis to ascertain the main source of infection for animals
- Evaluate the host specificity of *Giardia* and *Cryptosporidium* species from the study area

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ANNEXURE

Qualitative and Quantitative test (Willis and McMaster) as described by Ueno and Gonçalves (1998)

Willis technique

1. Weigh 2-5 grams of faeces and mix with approximately 20ml of NaCl solution
2. Homogenize and filter the solution
3. Put the solution within a small barrel cup
4. After 15 minutes the eggs are collected from the surface of the liquid onto a glass slide, which is then examined under the microscope.

McMaster technique

1. Suspend 2g of fresh faeces accurately weighed in 58 ml of NaCl solution.
2. Homogenize and filter the solution
3. With Pasteur pipet remove a small amount of solution and carefully apply in each compartments of the McMaster chamber.
4. Allow the camera to rest for 1-2 minutes and examine under optical microscope 10X
5. For the calculation of EPG multiplies the total number of the eggs found in both compartments by 50, and by 100 for one compartment.

Formol Ether concentration as described by Cheesbrough (1987)

1. Emulsify 4 ml of formol 10% in 1g of faecal sample, add 4 ml of formol v/v, shake for 20 seconds and sieve the suspension.
2. Transfer the suspension to conical tube (15ml), add 4 ml of ether and mix vigorously for 15 seconds and centrifuge at 3000 rpm for 1 minute
3. Decant the supernatant using pasteur pipette and mix the sediment

Modified Ziehl Neelsen technique as described by Cheesbrough (1987)

1. Prepare thin smear and fix in absolute methanol
2. Stain with carbol fuchsin for 10 minutes and wash with clean water
3. Decolorize using 3% hydrochloric acid in 95% ethanol, wash with clean water
4. Counterstain with 0,25% w/v malachite green for 30 seconds, wash with clean water
5. Let the slide to dry and examine the smear in optical microscope using 100x objective