An epidemiological study of cryptosporidiosis at the wildlife/livestock/human interface in Mpumalanga Province, South Africa

by

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DECLARATION

I, Nada Abu Samra, hereby declare that the work on which this thesis is based is original and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree at this or any other University.

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SIGNATURE   DATE

DEDICATION

To: Ferran, Mateo and Aran

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SUMMARY

An epidemiological study of cryptosporidiosis at the wildlife/livestock/human interface in Mpumalanga Province, South Africa

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Cryptosporidium spp. is an oocyst-forming apicomplexan protozoan, which infects humans and a large variety of animals. Several species and genotypes are potentially zoonotic and ruminants are considered as an important source of infection. Pre-weaned calves are major hosts for zoonotic *C. parvum*, and show higher rates of infection than post-weaned or adult animals. *Cryptosporidium* infection has been demonstrated in a wide variety of wild animals, which may contribute to environmental contamination. In sub-Saharan Africa, where the HIV infection prevalence is the highest in the world, high incidence of severe and even fatal *Cryptosporidium* infection have been reported in humans. This study investigated the epidemiology of *Cryptosporidium* spp. simultaneously in wildlife, indigenous cattle and young children living at the wildlife, livestock and human interface on the western boundary of the Kruger National Park (KNP) in Mpumalanga Province, South Africa.

Initially, a pilot study was carried out to assess the zoonotic or anthroponotic importance of *Cryptosporidium* in diarrhoeic children in South Africa, representing the human group most likely to be infected. This geographically broad study involved hospitals from four provinces in South Africa. Stool samples from hospitalized diarrhoeic children from 0-1 year of age were analysed by microscopy (modified Ziehl-Neelsen (MZN) acid-fast staining) and

molecular techniques: polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and sequencing. An overall prevalence of *Cryptosporidium* infection of 12.2% was revealed, mainly involving species of anthroponotic origin, such as *C. hominis* (76%) and *C. parvum* of anthroponotic nature (20%). Only one species of uncertain zoonotic importance (*C. meleagridis*) was detected in this study. These findings were in accordance with reports from sub-Saharan Africa (including South Africa), where anthroponotic species of *Cryptosporidium* were responsible for most infections in humans.

The study then concentrated on the public health importance of cryptosporidiosis at the wildlife/livestock/human interface of the KNP. *Cryptosporidium* oocysts were detected in elephant, impala and buffalo samples collected in three different study areas of the KNP; two located close to the boundaries of the KNP and a third one in the centre of the KNP. The MZN staining technique and an immunofluorescent antibody (IFA) test were applied to identify oocysts from faecal samples. The prevalence detected with MZN was higher than that detected with IFA, however both tests found a higher prevalence in elephants (25.8% and 4.2%, respectively) compared to the other species. The prevalence of *Cryptosporidium* in buffalo was 5.5% and 1.4% with MZN and IFA, respectively, and 4.2% and 1.8% respectively in impala. In the two study areas adjacent to the fence of KNP, the combined prevalence was significantly higher compared to the area in the centre of the KNP. The agreement between the MZN staining technique and the IFA test was assessed for each wildlife species; the estimates of kappa suggested moderate agreement in buffalo and impala and fair to poor agreement in elephant.

The above results of were analysed further by the use of molecular techniques in order to reveal the species and genotypes of the parasite in wildlife and in addition faecal samples collected from post-weaned calves. A questionnaire was also conducted among farmers to investigate observed contacts between cattle and wildlife species in grazing areas outside and inside the KNP. Four of the 241 wildlife samples were PCR-positive (2.8% each in impala and buffalo and of 0.0% in elephant) and sequencing revealed the presence of *C. ubiquitum* in two impala and one buffalo and *C. bovis* in one buffalo. *Cryptosporidium ubiquitum* has been commonly found in a large number of animals, including humans. Among calf samples, 8% (4/51) were PCR-positive and were identified as *C. andersoni* (2/4) and *C. bovis* (2/4). The probability of contact between cattle and wildlife outside the KNP, observed by farmers,

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was higher for buffalo (Pr=0.6) and impala (Pr=0.46) than for elephant (Pr=0.04). This suggests that the detection of *C. bovis* in both cattle and buffalo might be due to direct or indirect contact between these two species. The detection of *C. ubiquitum* in wildlife, with its zoonotic potential, suggests that *Cryptosporidium* may be of public health concern for people living at the interface.

We further investigated the prevalence of *Cryptosporidium* infection in cattle and humans, this time targeting younger (pre-weaned) calves and children. Children <5 years were sampled at six rural clinics within the same interface and stool samples were screened by the MZN staining technique. All MZN-positive and suspicious samples of children and samples of 36 calves within the age of 0-4 months were analysed by nested PCR. Eight of the 143 children (5.6%) were positive on PCR, and sequencing identified predominantly *C. hominis*, while one sample was identified as *C. meleagridis*. Eleven of the 36 calf samples (30.5%) were PCR-positive and were identified as *C. bovis* and *C. ryanae*. Due to limited resources, molecular analysis could not be performed on more samples. Variables such as source of drinking water, age and contact with animals for children, were analysed as potential risk factors for humans and cattle; however, none were statistically significant.

In conclusion, the prevalence of *Cryptosporidium* detected in human and wildlife was low compared to that reported in other studies in Africa. The species and genotypes detected in humans were predominantly of anthroponotic nature; however, the isolation of *C. Ubiquitum* from buffalo and impala shows that at least one species of zoonotic importance is present at the wildlife/livestock/human interface. The prevalence of HIV/AIDS in our study area is one of the highest worldwide; therefore the potential public health importance of this parasite should be investigated further.

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AIDS	Acquired immunodeficiency syndrome
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
COWP	Oocyst wall protein
CsCl	Caesium chloride
DNA	Deoxyribonucleic acid
ds	Double stranded
ELISA	Enzyme-linked immunosorbent assay
GP60	60k Da glycoprotein
HIV	Human immunodeficiency virus
HSP70	70k Da heat shock protein
IFA	Immunofluorescence antibody
KNP	Kruger National Park
MZN	Modified Ziehl-Neelsen
MTA	Mnisi Traditional Authority
NICD	National Institute for Communicable Diseases
PCR	Polymerase chain reaction
PRU	Parasitology Unit
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rt PCR	Real time PCR
SSU	Small subunit
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

Cryptosporidium is a coccidian protozoan parasite of the phylum Apicomplexa, class Sporozoa, subclass Coccidia, order Eucoccidiida, suborder Eimeriina and family Cryptosporidiidae (Carmena, 2010). To date, 26 *Cryptosporidium* species have been recognized (Chalmers and Katzer, 2013) and nearly 50 genotypes have been described (Xiao and Feng, 2008a), while new genotypes are continually being discovered (Feng et al., 2007a).

Cryptosporidium has the ability to infect a large variety of animals, including humans, worldwide. Humans can acquire *Cryptosporidium* infections through direct contact with infected persons (anthroponotic transmission) or animals (zoonotic transmission) via ingestion of contaminated food (foodborne transmission) or water (waterborne transmission) (Xiao, 2010). Young individuals appear to be more susceptible to infection and disease, while infections in adults are often asymptomatic. Therefore cryptosporidiosis occurs most commonly in young animals and children in association with yellow watery diarrhoea which causes dehydration, weight loss, fever, and inappetence (O'Donoghue, 1995; Ramirez et al., 2004). In immunocompetent hosts these symptoms are usually self-limiting, while in immunosuppressed individuals, such as Human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) patients, chronic diarrhoea may last for more than 2 months and can lead to mortality (Chalmers and Davies, 2010).

Since *Cryptosporidium* is a diarrhoeal disease of worldwide importance in humans, especially young children and immunosuppressed individuals, it is part of the complex group of parasitic, bacterial and viral diseases included in the World Health Organisation's *Neglected Disease Initiative* (WHO, 2004). In developing regions of the world, these diseases are not adequately addressed nationally and internationally and cause considerable socio-economic burden in poor populations, particularly in remote and rural areas (Putignani and Menichella, 2010; Savioli et al., 2006).

The life cycle of most *Cryptosporidium* species is completed within the gastrointestinal tract of the host. Infective (thick-walled) oocysts are excreted in the faeces and are able to survive for long periods outside the host in a moist ~~ambient~~ environment, and may remain viable in water for 140 days (Putignani and Menichella, 2010; Ramirez et al., 2004) . In addition, they are very resistant to the most common disinfectants, making them difficult to

destroy by chlorination treatment (Ramirez et al., 2004). A new host becomes infected by ingesting these oocysts, which release sporozoites, primarily in the small intestine and colon. At this point the sporozoites invade the epithelial cells and undergo asexual and sexual multiplication to produce thin-walled and thick-walled oocysts. The thin-walled oocysts have the ability to excyst endogenously and infect new cells, which leads to autoinfection. Due to the rapidity of the life cycle, along with the auto-infective cycle, huge numbers of organisms can ~~colonize~~ infect the enterocysts ~~the intestinal tract~~ within several days. The excreted thick-walled oocysts are fully sporulated and immediately infective (Chako et al., 2010). The duration of oocyst shedding can range from several days to months or years, depending on the host's immunocompetence (Ramirez et al., 2004).

DIAGNOSIS OF CRYPTOSPORIDIOSIS

Conventional methods for diagnosis of *Cryptosporidium* infection in humans and animals are based on microscopic examination of stained faecal samples. Modified Ziehl-Neelsen (MZN) staining technique is still considered the gold standard for the detection of *Cryptosporidium* oocysts (Potters and Van Esbroeck, 2010). However, the sensitivity and specificity of the MZN technique in faecal samples from humans are reported to be 83.8% and 98.9%, respectively (Fayer et al., 2000; Morgan et al., 1998; Paul et al., 2009), and 79.3% and 67.7%, respectively in samples from cattle and pigs (Quílez et al., 1996a). The concentration of stool samples, using Sheather's sugar flotation or the formalin-ethyl acetate sedimentation method, prior to staining has been described as essential since it allows the detection of seven times fewer oocysts than unconcentrated stool samples (Paul et al., 2009; Quílez et al., 1996a). The MZN technique is cost-effective; however, staining is a time consuming procedure (about 30 to 45 minutes) that requires intensive training and experience to interpret the results. A common problem is distinguishing *Cryptosporidium* oocysts from other elements, such as moulds and yeast (Potters and Van Esbroeck, 2010).

Monoclonal antibody-based immunofluorescence assays (IFA) offer an alternative method to conventional staining techniques and have been shown to be more sensitive for the detection of *Cryptosporidium* oocysts, especially in human and animal stools that contain

few parasites and large amounts of debris (Garcia and Shimizu, 1997; Quílez et al., 1996a). In general, microscopic techniques have limited sensitivity and specificity. Therefore, their application for generation of prevalence data in epidemiological investigations has been questioned, whereas for clinical purposes microscopy techniques are believed to be sufficient, since the number of oocysts excreted by symptomatic patients is high (Quílez et al., 1996a).

Molecular techniques, such as polymerase chain reaction (PCR), can ensure specific diagnosis to species/genotype (Coupe et al., 2005; Xiao et al., 1999) and subtype level (Xiao, 2010). Only a few genes have been characterized for various *Cryptosporidium* spp., such as the small subunit rRNA (SSU rRNA) or 18S rRNA gene, 70kDa heat shock protein (HSP70) and the oocyst wall protein (COWP). Use of the SSU rRNA gene has some advantages over other genes because of the higher copy numbers and the presence of conserved regions interspersed with highly polymorphic regions, which facilitate the design of PCR primers (Xiao et al., 2004). Several PCR-restriction fragment length polymorphism (RFLP) techniques have been described for the differentiation of *Cryptosporidium* spp., all based on the SSU rRNA (Xiao and Ryan, 2004). Subgenotyping tools have been developed for *C. hominis* and *C. parvum*: microsatellite tools, HSP70, a double stranded (ds) RNA, and 60 kDa glycoprotein (GP60) gene tools (Xiao et al., 2001). The GP60 gene has a high variation in the number of trinucleotide repeats with extensive sequence differences in the non-repeat regions, which categorizes *C. parvum* and *C. hominis* into several subtype families, namely Ia, Ib, Id, Ie, If and Ig for *C. hominis* and IIa –IIi, IIk and III for *C. parvum* (Xiao, 2010).

ZOONOTIC CRYPTOSPORIDIOSIS

Cryptosporidium has been known to be a zoonotic disease for some time, but its zoonotic significance was only realised in the 1980s, when infections were reported to be a cause of death in HIV/AIDS patients (Xiao and Feng, 2008a). Cattle are considered to be an important source of zoonotic cryptosporidiosis; several outbreaks in humans have been associated with infected calves, and small cryptosporidiosis outbreaks have occurred in veterinary students (Gait et al., 2008), research technicians(Kiang et al., 2006), and children attending agricultural camps and fairs (Smith et al., 2004). *Cryptosporidium* infection has also been

found in a wide range of wild animals, representing a potentially significant source of environmental contamination and reservoir of the disease for domestic livestock and humans (Feng et al., 2007a; Hunter and Thompson, 2005).

Once *Cryptosporidium* oocysts of animal or human origin are released into the environment they may be transmitted via contaminated water or food, causing severe diarrhoea in both humans and animals (Putignani and Menichella, 2010). Several outbreaks of zoonotic and anthroponotic origin have been associated with contaminated surface water, well water, swimming pools and public water supplies. One of the largest human waterborne outbreaks occurred in Milwaukee, Wisconsin, USA in 1993, in which more than 400,000 people were infected (Mac Kenzie et al., 1994). Drinking Milwaukee municipal water, supplied by a water treatment plant, was identified as the source of the outbreak, while the source of transmission (zoonotic or anthroponotic) remains unknown (Osewe et al., 1996). Since then, *Cryptosporidium* became of great public health interest and has been recognized as one of the most serious and difficult waterborne pathogens to control (Ramirez et al., 2004).

The role of animals, wild and domestic, in the transmission of human cryptosporidiosis is not entirely clear, mainly because the oocyst morphology in human-pathogenic and non-human-pathogenic species is similar (Paul et al., 2009). The use of molecular tools has therefore contributed to a better understanding of the sources and causes of *Cryptosporidium* outbreaks, the zoonotic potential of various *Cryptosporidium* species and the role of animals in the transmission of human cryptosporidiosis (Ramirez et al., 2004; Xiao and Feng, 2008a). For example, in 2003 in Ohio (USA), an outbreak of cryptosporidiosis was associated with apple cider and molecular analysis of faecal samples of affected patients and of samples of the partially drunk cider, revealed the presence of *C. parvum*, belonging to the same subtype family, in both samples. The *C. parvum* subtypes found in this outbreak were frequently found in cattle on farms in the same area (Blackburn et al., 2006).

Much attention has been given to zoonotic *C. parvum*; however, not all *C. parvum* subtypes are of zoonotic origin. Recent subtyping and multilocus typing studies have revealed that the source of *C. parvum* in humans can be of bovine origin (zoonotic) (Alves et al., 2006; Feltus et al., 2006; Gatei et al., 2002; Glaberman et al., 2002) or human-adapted

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(anthroponotic)(Cama et al., 2007; Leav et al., 2002b; Morse et al., 2007; Peng et al., 2003a); six *C. parvum* subtypes (IIb, IIc, IIe, IIf, IIh and IIi) have never been found in animals and are therefore considered to be anthroponotic. In contrast, *C. parvum* subtypes IIa, IId and III have been responsible for zoonotic infection, detected in humans and animals living in the same area (Alves et al., 2006; Gait et al., 2008; Helmy et al., 2013; Sulaiman et al., 1998). Among different geographic areas diverse *C. parvum* subtypes have been detected: many of the common bovine IIa subtypes are dominant *C. parvum* subtypes in humans in North America, Europe and Australia, while bovine subtype IId has been reported to be responsible for some infections in Europe and III has only been found occasionally in Europe. On the contrary, in developing countries the transmission of *C. parvum* in humans is mostly anthroponotic and *C. parvum* subtype IIc has predominantly been reported (Xiao, 2010).

To study the zoonotic potential of *C. parvum* in farm animals, especially calves, gp60 sequence analyses have been done, mostly in industrialized nations (Xiao, 2010). Results of these studies show that calves are mainly infected with the IIa subtype family, which has been described from Hungary (Plutzer and Karanis, 2007), Slovenia (Soba and Logar, 2008), Serbia (Misic and Abe, 2007), the United Kingdom (Brook et al., 2009), , and Australia (Ng et al., 2011). In European countries the *C. parvum* subtypes IId and III have been occasionally found in calves; several IId subtypes have been found in dairy calves in Portugal (Alves et al., 2006), Spain (Quilez et al., 2008), Hungary (Plutzer and Karanis, 2007), Germany (Broglia et al., 2008), Belgium (Geurden et al., 2007) and Serbia (Misic and Abe, 2007). In African and Asian countries *C. parvum* subtype IIa and IId have been reported in bovine and buffalo calves in Egypt (Amer et al., 2013; Helmy et al., 2013), in cattle and humans in Iran (Nazemalhosseini-Mojarad et al., 2011), and in preweaned calves in China (Wang et al., 2011) and Malaysia (Muhid et al., 2011).

Likewise, III subtypes were found in a few calves in Slovenia (Soba and Logar, 2008) and Serbia (Misic and Abe, 2007). Young, pre-weaned calves, particularly under 2 months of age, are of greatest public health significance in spreading *C. parvum*, since they are more commonly infected than post-weaned calves; in a study conducted in the US, *C. parvum* was found in 85% of pre-weaned calves and only in 1% of post-weaned calves (Santín et al., 2004). However, molecular characterization of *Cryptosporidium* spp. has been conducted in

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native breeds of cattle in Nigeria and this study failed to demonstrate the presence of *C. parvum* in pre-weaned calves reared in traditional husbandry systems (Maikai et al., 2011). It was therefore suggested that native breeds of cattle may not be important in the transmission of cryptosporidiosis to humans in developing countries.

CRYPTOSPORIDIOSIS IN WILDLIFE

The significance of wildlife as a reservoir for farm animals or humans is uncertain, although *Cryptosporidium* has been demonstrated in a wide range of wild animals. Contamination of water and pasture could be responsible for direct contamination to domestic animals and humans. Similarly, environmental pollution with human or domestic animal faecal material could be a potential pathway for infection of wildlife with zoonotic *Cryptosporidium* species. The prevalence of *Cryptosporidium* in wild animals varies globally and at least 11 *Cryptosporidium* species and nearly 30 genotypes are known to infect wild animals (Feng, 2010). In eastern and sub-Saharan Africa, *Cryptosporidium* oocysts have been described in Mikumi National Park in Tanzania, where 28% of African buffalo, 28% of zebra and 27% of wildebeest were found to be infected (Mtambo et al., 1997), and in Uganda a prevalence of 11.1% was detected in non-human primates (Salyer et al., 2012). *Cryptosporidium* species have been found in various species of deer in the USA and Canada, with prevalence in white-tailed deer between 0.2% and 11% and in red deer between 0.3% and 26.9% (Feng et al., 2007a). *Cryptosporidium* oocysts have also been reported in captive animals such as lowland anoa, prairie bison, bongo, greater kudu, gemsbok, tule elk, sable antelope, caribou, impala, springbok and oryx, with prevalences between 3.3% and 26.9% (Feng, 2010). However, recent genetic studies indicate the presence of mainly host-adapted species and genotypes in wild animals, suggesting limited potential for cross-species transmission and limited public and veterinary health importance (Feng, 2010). Host-adapted genotypes have also been reported in rodents, such as the deer mouse genotypes I, II, III and IV in deer mice (Feng et al., 2007a; Xiao et al., 2002), the muskrat genotypes I and II in muskrats and voles (Feng et al., 2007a; Zhou et al., 2004), and the squirrel genotype in ground squirrels (Atwill et al., 2004; Xiao et al., 2004). In carnivores, the bear genotype was reported in black bears, the ferret genotype in ferrets, and the skunk genotype in skunks,

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raccoons and river otters; foxes are known to be infected with multiple *Cryptosporidium* species and genotypes, such as *Cryptosporidium* fox genotype, *C. canis* fox subtype and *C. canis* dog subtype (Xiao et al., 2002; Zhou et al., 2004) . In white-tailed deer, the cervine genotype and the deer genotype have been reported (Feng et al., 2007b; Karanis et al., 2007; Ryan et al., 2003; Wang et al., 2008; Xiao et al., 2002).

Nevertheless, non-species-specific *Cryptosporidium* species with potential zoonotic importance have been described in wildlife, albeit in a limited number of species. *Cryptosporidium parvum* infection has been reported in a few rodents, grey wolves, raccoon dogs, white-tailed deer and captive mountain gorillas (Feng, 2010). *Cryptosporidium andersoni* was found in a European wisent (Ryan et al., 2003), *C. meleagridis* was reported in a deer mouse (Feng et al., 2007a), and *C. canis* dog genotype was reported in one fox (Xiao et al., 2002). *Cryptosporidium ubiquitum* has been reported in several wildlife species: in the deer mouse, eastern gray squirrel, red squirrel, eastern chipmunk, North American beaver, woodchuck, raccoon, white-tailed deer and sika deer, as well as in captive sika deer, blesbok and nyala (Feng et al., 2007a; Karanis et al., 2007; Ryan et al., 2003; Wang et al., 2008). Because of its broad host range, Feng (Feng, 2010) concluded that it is probably the species of greatest public health concern.

CRYPTOSPORIDIOSIS IN CATTLE

Cryptosporidiosis most commonly affects neonatal calves and is considered one of the most common enteropathogens found in calves during the first weeks of life. Profuse watery diarrhoea is the primary clinical sign and contributes to high mortality rates, which may lead to economic losses (Chako et al., 2010; de Graaf et al., 1999). Drugs demonstrated to be only partially effective in the treatments and prophylaxis, at recommended dosages. Halofuginone at an oral dose rate of 0.1 mg/kg bodyweight daily verified to be effective treatment for cryptosporidial diarrhoea, but its efficiency in reducing oocyst shedding has not been demonstrated. Therefore disease control by preventive hygiene measures are the most important tools in the struggle against cryptosporidiosis in farm animals (Chako et al., 2010).

Cryptosporidiosis in cattle has been reported worldwide (Coklin et al., 2007; Fayer et al., 2006; Joachim et al., 2003; Maddox-Hyttel et al., 2006; Quílez et al., 1996b). In African countries, the prevalence of *Cryptosporidium* infection in smallholder dairy cattle in Tanzania was 19.7% (Swai et al., 2007), in Uganda a prevalence of 38% was detected in calves (Nizeyi et al., 2002), and in Kenya a prevalence of 20% was detected in cattle from dairy-keeping households in urban settings (Kang'ethe et al., 2012). In Zambia, the prevalence in calves in dairy, beef and traditional husbandry systems was 42.8%, 8.0% and 6.3%, respectively (Geurden et al., 2006) and in native breeds of calves from traditional husbandry systems in Nigeria a prevalence of 16% was reported (Maikai et al., 2011).

The prevalence of *Cryptosporidium* infection in cattle is highly age-dependent, with the highest prevalence (28%) in unweaned calves (Brook et al., 2008), compared to 2.4% in weaned calves (calves > 6 months) (Wade et al., 2000). In addition to the age-related variation in prevalence, an age-related occurrence of different *Cryptosporidium* species has been reported: *C. parvum* is mostly found in pre-weaned (5 days to 2 months) dairy calves, *C. bovis* and *C. ryanae* in post-weaned (3-11 months) calves, and *C. andersoni* in yearlings and adult cattle (Xiao, 2010).

CRYPTOSPORIDIOSIS IN HUMANS

Cryptosporidiosis is recognized globally as an important cause of diarrhoea in children and adults. Most cases are detected in children under the age of 5 years, and mainly in those <2 years of age, in both developing and developed countries (Xiao and Feng, 2008a). Although clinical manifestations of cryptosporidiosis vary with age and immune status, they include profuse diarrhoea of 1-14 days duration, often accompanied by abdominal cramps, vomiting, fever and malaise (Cama et al., 2008b; Chako et al., 2010). In immunosuppressed people, *Cryptosporidium* infection can cause chronic and/or life-threatening diarrhoea (Caccio, 2005). In sub-Saharan Africa, where HIV prevalence rates are high, people are at greater risk for clinical cryptosporidiosis, especially considering poor hygienic practices, which favour water and foodborne transmission (WHO, 2008).

No drugs are available that effectively treats cryptosporidiosis in humans. Macrolides, paromycin and nitazoxanide have partial efficacy in reducing disease severity in immunocompetent individuals (Mead, 2002) and in HIV positive patients anti-retroviral treatment reduces clinical manifestations. However it has been recommended by the World Health Organization (WHO) that the main preventive strategy against cryptosporidiosis in sub-Saharan Africa is boiling of drinking water (WHO, 2008).

On the African continent, the reported prevalence of *Cryptosporidium* varies widely. In Nigeria a prevalence of 1.9% was reported in patients aged 2 months to 70 years (Maikai et al., 2012) and a prevalence of 32.4% was detected in humans living in the region of Kibale National Park, Uganda (Salyer et al., 2012). In Equatorial Guinea a prevalence of 18.9% was detected in hospitalized patients of which 92% were HIV-positive (Blanco et al., 2009) and in Ethiopia a prevalence of 7.6% was found in diarrhoeic patients (Adamu et al., 2010). In Kenya a prevalence study on *Cryptosporidium* was carried out among cattle-keepers and their non-cattle-keeping neighbours in the dry and wet seasons, and among HIV-positive patients (Kange'the et al., 2012). Among dairy-keeping family members, 4% were found to be infected during the dry season and 0.3% during the wet season; in non-dairy households prevalences of 5% and 0% were detected, respectively; the prevalence in HIV-positive patients was 5% in both seasons. In South Africa a prevalence of 24.8% was found in HIV-positive diarrheic children of one hospital in Durban, KwaZulu-Natal, (Leav et al., 2002b), and a prevalence of 18% has been reported from the Venda region, Limpopo Province, among school children and hospital patients (Samie et al., 2006).

Cryptosporidium parvum and *C. hominis* are responsible for more than 90% of human cases of cryptosporidiosis, with *C. meleagridis*, *C. felis*, *C. ubiquitum* and *C. canis* responsible for most of the remaining cases (Xiao, 2010). A few other *Cryptosporidium* genotypes are occasionally found in humans, including *C. muris*, *C. suis*, *C. andersoni*, and the *Cryptosporidium* cervine, skunk, and chipmunk I genotypes. *Cryptosporidium ubiquitum* has been reported in faeces from humans over a wide geographic range: in Canada, New Zealand, Slovenia, United Kingdom and the USA (Xiao and Feng, 2008a), but not, to our knowledge, in Africa.

The prevalence of *C. parvum* and *C. hominis* varies in different geographic regions; in European countries *C. parvum* and *C. hominis* are both common in humans, in the Middle East *C. parvum* is the predominant species in humans, and in developing countries *C. hominis* is usually the predominant species in humans (Xiao, 2010). In addition, the distribution of *C. parvum* and *C. hominis* can also vary within a country; for example, *C. parvum* is more common than *C. hominis* in rural settings of the United States and Ireland (Feltus et al., 2006; Zintl et al., 2009).

In developing countries, four common *C. hominis* subtype families, namely Ia, Ib, Id and Ie, are usually found in humans. In Peru, Malawi, Madagascar and India, all four common subtype families were seen in children and HIV positive adults (Ajjampur et al., 2007; Cama et al., 2008b; Gatei et al., 2007; Muthusamy et al., 2006; Peng et al., 2003c), and in South Africa in addition to these, the subtype family If has been reported in some children (Leav et al., 2002b). This high complexity of *C. hominis* in developing countries is likely an indicator of intensive and stable cryptosporidiosis transmission in these areas (Xiao, 2010).

OUTLINE OF THE THESIS

South Africa is known for its large protected wildlife areas, in particular the KNP and its surrounding private game reserves. A large number of rural communities with a high concentration of people and their livestock live on the periphery of these protected areas, sometimes sharing grazing areas and water sources with wild animals. Since the prevalence of HIV infection in rural communities is high and the boundaries of the protected areas are permeable, this environment creates a suitable situation for the circulation of pathogens between wildlife, livestock and humans. In this scenario, neglected zoonotic diseases, such as cryptosporidiosis, should be considered a high priority for further study. The general objective of the studies presented in this thesis was to evaluate the zoonotic importance of *Cryptosporidium* at the wildlife/livestock/human interface along the boundary of the KNP in Mpumalanga Province, South Africa.

More specifically, the objectives were to gain insight into the epidemiology of cryptosporidiosis at the interface by determining the prevalence of infection in the different

compartments (wildlife, livestock and humans), identifying the species and genotypes present in each compartment, and investigating possible risk factors for infection. Therefore the following research questions were addressed:

1. Is cryptosporidiosis an important zoonotic disease in South Africa?

2. Is *Cryptosporidium* circulating in free ranging wildlife in the KNP and adjacent protected areas and, if so, at what level?

3. What species and genotypes of *Cryptosporidium* occur in wildlife and livestock at the edge of the protected area, and is there evidence for transmission between the two compartments?

4. Is *Cryptosporidium* an important zoonosis in human communities living close to the KNP?

The first research question was addressed in Chapter 2 of this thesis, in which a geographically broad study on the presence of *Cryptosporidium* spp. in children from four provinces in South Africa is described. This study provided information on the prevalence of cryptosporidiosis in the most vulnerable population (children) and in those most likely to be infected (those with diarrhoea), and identified the species and genotypes present. The potential zoonotic and anthroponotic importance of the disease is discussed.

Chapter 3 addressed the second research question, describing the prevalence of *Cryptosporidium* infection in three of the most common large wildlife species present in the KNP: African elephant, impala and African buffalo. Animals were sampled at different locations, close to potential livestock contact on the boundary of the KNP and remote from any livestock contact in the centre of the park. In this study *Cryptosporidium* spp. oocysts were detected by conventional microscopy (MZN and IFA) and confirmed by PCR.

In Chapter 4, with the use of molecular techniques, *Cryptosporidium* species and subtypes were identified in wildlife, using the same samples described in Chapter 3, as well as in additional samples from cattle living in close proximity to the KNP. This chapter addressed

the third research question, investigating the possible presence of zoonotic *Cryptosporidium* species in animals at the wildlife/livestock/human interface.

Chapter 5 addressed the fourth research question, as well as aspects of the first and third research questions, and was conducted in a different study area, also along the western boundary of the KNP. This chapter reports a study of *Cryptosporidium* species and subtypes in cattle and humans living at the wildlife/livestock/human interface, focusing on young age groups including pre-weaned calves and children less than 5 years of age. It investigated the presence of zoonotic *Cryptosporidium* species in order to better understand the role of animals in the transmission of cryptosporidiosis to humans, and also attempted to identify potential risk factors for infection in humans and cattle.

Finally, in the General Discussion in Chapter 6, the findings with respect to research questions 1 to 4 were discussed, interpreted and compared to existing knowledge published in the literature. Further combined analysis of results from the four preceding chapters was done in order to better understand the performance of the diagnostic tests used, the limitations of the study were discussed, and recommendations for further research were made.

During the course of this study the opportunity arose to test some of our cattle and wildlife samples for one microsporidian species, *Enterocytozoon bieneusi*, commonly found in humans and animals. The modes of transmission of this parasite are comparable to *Cryptosporidium* and therefore the findings of this study were relevant to this thesis and the resultant published paper is included as an addendum (Appendix 1). Both parasitic diseases are leading causes of chronic diarrhoea and may be of public health importance, especially in areas with poor ~~sanitation~~ sanitary conditions.

CHAPTER 2

GENETIC CHARACTERIZATION OF *CRYPTOSPORIDIUM* SPP. IN DIARRHOEIC CHILDREN FROM FOUR PROVINCES IN SOUTH AFRICA

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ABSTRACT

The diversity of *Cryptosporidium* at species, subtype family and subtype level in diarrheic children was investigated in four provinces in South Africa. A total of 442 stool samples from children less than 5 years of age was collected under a large rotavirus surveillance programme and analyzed by modified Ziehl-Neelsen (MZN) staining technique. Fifty four (12.2%) were positive for *Cryptosporidium*, of which 25 were genotyped by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) and DNA sequence analyses of the 18S rRNA gene. The majority of genotyped specimens were identified as *C. hominis* (76%) and a high genetic diversity was found with five different *C. hominis* subtype families (Ia, Ib, Id, Ie and If). *Cryptosporidium parvum* was found in 20% of the isolates and three subtype families were identified (IIc, IIe and IIb), with subtype family IIc being the most common. One specimen was identified as *C. meleagridis* of the subtype family IIId. These results are in accordance with findings from other developing countries, and report for the first time the presence in South Africa of *C. meleagridis*, various subtypes of *C. parvum*, and the subtype family Ie of *C. hominis*. The results suggest that *C. hominis* and anthroponotic *C. parvum* subtypes are the major cause of cryptosporidiosis in South Africa. Further molecular studies are needed to better understand the epidemiology and public health importance of *Cryptosporidium* in humans in South Africa.

INTRODUCTION

Cryptosporidium is an enteric parasite in a wide range of hosts, including humans, domestic and wild animals. *Cryptosporidium* infection can be associated with profuse, chronic, or even life-threatening diarrhoea, particularly in young children and in immunocompromised persons (Xiao and Ryan, 2004). Children younger than 2 years of age are frequently infected in community and hospital settings in developing countries (Bern et al., 2000; Cama et al., 2008a). Humans can acquire *Cryptosporidium* infections through direct contact with infected persons (anthroponotic transmission) or animals (zoonotic transmission), or by ingestion of contaminated food (foodborne transmission) or water (waterborne transmission) (Xiao, 2010).

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The use of molecular tools to genotype and subtype *Cryptosporidium* parasites has contributed to improved understanding of the transmission of cryptosporidiosis in humans and animals. Humans can be infected by at least 10 *Cryptosporidium* species (*C. hominis, C. parvum, C. meleagridis, C. canis, C. felis, C. cuniculus, C. suis, C. muris, C. andersoni,* and *C. ubiquitum*) and four genotypes (horse, skunk, chipmunk genotype I, and pig genotype II), with *C. hominis* and *C. parvum* responsible for most infections (Xiao, 2010).

Molecular characterization of the 60-kDa glycoprotein (gp60) gene has recently been used for *C. hominis* and *C. parvum* subtyping. These analyses have shown at least eight *C. parvum* subtype families: two zoonotic (IIa and IId) and six anthroponotic (IIb, IIc, IIe, IIf, IIh, and IIi) ones (Peng et al., 2003b; Sulaiman et al., 2005b). Within each subtype family several subtypes have been described based on variations in the number of trinucleotide repeats (TCA, TCG or TCT) of the gp60 gene (Xiao, 2010).

Geographic differences in the distribution of *C. parvum* and *C. hominis* infections have been reported: in European countries both *C. parvum* and *C. hominis* are responsible for significant numbers of human infections, in the Middle East *C. parvum* is the dominant species in humans, while in developing countries the predominant species is *C. hominis* (Xiao, 2010). The anthroponotic IIc subtypes of *C. parvum* are responsible for most human infections in developing countries (Akiyoshi et al., 2006; Leav et al., 2002a; Peng et al., 2003b; Xiao and Feng, 2008b); however in some African countries such as in Malawi and Kenya, unusual anthroponotic *C. parvum* subtype families (IIe and IIb) have been found in addition to the most common IIc subtype family (Cama et al., 2007; Peng et al., 2003b; Xiao and Ryan, 2004). Furthermore, in developing countries a high genetic diversity among *C. hominis* has been observed, most commonly Ia, Ib, Id, Ie and If (Cama et al., 2007; Gatei et al., 2007; Leav et al., 2002a; Peng et al., 2003b).

In southern Africa, the number of molecular epidemiological studies of cryptosporidiosis in humans is very limited. Ten years ago, a study was carried out in South Africa, where 20 specimens from hospitalized children with diarrhoea in Durban, KwaZulu-Natal Province, were genetically characterized by gp60 sequence analysis; the majority of these samples were identified as *C. hominis* with four different subtype families Ia, Ib, Id and If, and a small number was observed as *C. parvum* with one subtype family (IIc) (Leav et al., 2002b). More

recently, another study, based on PCR-RFLP analysis of the 18S rRNA gene in children and elderly persons from the Vhembe district (Limpopo Province), observed *C. hominis* in most infected individuals (82%; 36/44) and *C. parvum* in a smaller proportion (18%; 8/44) (Samie et al., 2006). However, there are no published studies from South Africa describing the circulation of *Cryptosporidium* spp. on a wider geographic scale.

In this study, we analysed the diversity of *Cryptosporidium* at the species, subtype family and subtype levels in diarrheic children of 0-12 months in age, from four different provinces in South Africa (Cohen et al., 2010). The findings of this study were compared with those conducted in South Africa and other developing countries, particularly those on the African continent.

MATERIALS& METHODS

SAMPLING

The study formed part of a large rotavirus surveillance programme carried out at four sites in South Africa (Cohen et al., 2010). The programme aimed to estimate the number of hospitalisations due to severe diarrhoea in children. Patients of less than 5 years of age were sampled and tested for diarrhoea-causing viruses, bacteria and parasites. Stool samples were collected with parental consent by study nurses over a period of 10 months (June 2010 to April 2011) and were tested for rotavirus using an Elisa kit (Oxoid, UK) and PCR confirmation (Cohen et al., 2010). Any raw faecal material left after rotavirus testing was sent to the Parasitology Reference Unit (PRU) at the National Institute for Communicable Diseases (NICD) for detection of parasitic causes of diarrhoea.

A total of 442 specimens were available for this study.One hundred and forty-one samples were from the Chris Hani Baragwanth Hospital (Gauteng Province), 128 from the Agincourt hospitals (Mpumalanga Province), 147 from the Dr. George Mukhari Hospital (North West Province), and 26 from the Edendale Hospital (KwaZulu-Natal Province) (Fig. 1).
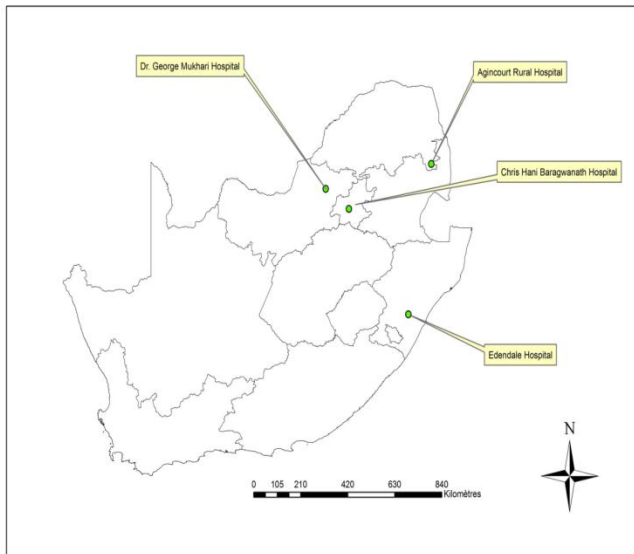
Figure 1: Location of clinics where sampling took place.

Samples were transported to the NICD for testing, where they were diluted with potassium dichromate (2.5%) and stored at room temperature until analysed. The same samples were tested within one week using MZN technique. Fifty-four samples were *Cryptosporidium*-positive and among those forty (all from children under 12 months of age) were available for molecular analysis.

DNA EXTRACTION AND *CRYPTOSPORIDIUM* GENOTYPING AND SUBTYPING

Molecular analysis was carried out at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, within fourteen months of receipt. DNA was extracted using the FastDNA SPIN kit for soil (BIO 101, Carlsbad, CA, USA). Briefly, approximately 0.5 g of faecal sample was transferred into a 2 ml tube containing lysing matrix E. After adding 100 µl sodium phosphate buffer and 122 µl MT buffer, the tube was vortexed in a FastPrep instrument (BIO 101). The sample was processed further following the manufacturer's suggested protocol. Initially, all specimens were genotyped by a PCR-restriction fragment length polymorphism (RFLP) technique in which an approximately 830-bp fragment of the 18S rRNA gene was amplified by a nested PCR. Primers and amplification conditions used in this study have been described previously (Xiao et al., 2001). Genotype identification was

made by restriction digestion of the secondary PCR product with the enzymes SspI and VspI (Xiao et al., 2001).

Cryptosporidium parvum and *C. hominis* were further subtyped by a nested PCR-sequence analysis of ~ 850 bp fragment of gp60 gene locus (Sulaiman et al., 2005a). All secondary PCR products were sequenced, using the forward and reverse primer used in secondary PCR and an intermediary sequencing primer (5'-GAGATATATCTTGTTGCG-3'), and the ABI BigDye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were read on an ABI1300 Genetic Analyzer (Applied Biosystems).The nucleotide sequences obtained in this study were assembled using the ChromasPro software (www.technelysium.com.au/ChromasPro.html) and aligned with *Cryptosporidium* reference sequences using ClustalX software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Subtypes were named by an established terminology (Sulaiman et al., 2005a; Xiao, 2010).

RESULTS

Of the 442 specimens tested by MZN technique, 54 (12.2%) were positive for *Cryptosporidium* spp. The highest prevalence was found at the Chris Hani Baragwanath Hospital (17.7%; 25/141), followed by Edendale Hospital (15.4%; 4/26), Dr. George Mukhari Hospital (9.5%; 14/147), and Agincourt (8.6%; 11/128). Due to the insufficient amount of faecal material, of these 54 samples, only 40 specimens were available for molecular analysis. Thirty samples were positive using 18S PCR and gp60 PCR and 25 samples were successfully subtyped at the gp60 locus. Sequence analysis of the gp60 locus identified *C. hominis* (76%; 19/25) most frequently, followed by *C. parvum* (20%; 5/25) and *C. meleagridis* (4%; 1/25). Five subtype families were identified within *C. hominis*: Ia, Ib, Id, Ie and If. The most common *C. hominis* subtype families observed were If and Id (5/19 each), followed by Ie (4/19), Ia (3/19), and Ib and Id (1/19 each). The highest diversity of *C. hominis* subtypes was found within subtype family Id with 4 subtypes, followed by Ia with 3 subtypes, Ib and If, each with 2 subtypes and Ie with one subtype (Table 2).

Infections with *C. parvum* belonged to three subtype families: IIc, IIe and IIb and the subtype family IIc was the most commonly found. In this study, only one isolate was identified as *C. meleagridis*, of the subtype family IIId and the subtype IIIdA4 (Table 2).

Table 1: *Cryptosporidium* species identified in diarrhoeic children in hospitals in South Africa

	C. parvum	*C. hominis*	*C. meleagridis*	TOTAL
Chris Hani Baragwanath (GP)	1	8	1	10
Agincourt (MP)	3	3	0	6
Dr. George Mukhari (NW)	1	7	0	8
Edendale (KZN)	0	1	0	1
Total	5	19	1	25

GP = Gauteng Province, MP = Mpumalanga Province, NW = North West Province, KZN = KwaZulu-Natal Province

Table 2: *Cryptosporidium* gp60 subtype families and subtypes identified in diarrhoeic children in hospitals in South Africa

Cryptosporidium species	gp60 subtype family	gp60 subtype	No. of isolates
C. hominis	Ia	Ia A20R3	1
(5 subtype families)		Ia A25G1R3	1
		Ia A17R3	1
	Ib	Ib A9G3	1
		Ib A10G1	1
	Id	Id A20	1
		Id A25	1
		Id A26	2
		Id A24	1
	Ie	Ie A11G3T3	4
	If	If A14G1	3
		If A12G1	2
Subtotal	**5**	**13**	**19**
C. parvum	IIb	IIb A11	1
(3 subtype families)	IIc	IIc A5G3b	3
	IIe	IIe A12G1	1
Subotal	**3**	**3**	**5**
C. meleagridis	IIId	IIId A4	1
Total	**9**	**17**	**25**

DISCUSSION

The present study produced data on the importance of *Cryptosporidium* in children with diarrhoea and the predominant *Cryptosporidium* species and subtypes circulating in this patient population in several provinces of South Africa. This is the third study on genetic characterization of *Cryptosporidium* spp. in children in South Africa and the first one providing information on a wide geographic scale.

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Only 25 of the 40 specimens analysed were positive in the 18S rRNA-based PCR. The number of false positive specimens in our study appear to be considerably higher than reported in literature; It has been demonstrated that PCR reactions improved the sensitivity to detect *Cryptosporidium* compared to MZN technique showed 83.7% sensitivity and 98.9% specificity compared to 100% sensitivity and specificity for PCR (Morgan et al., 1998). This discrepancy with our study may have been due to false positives on MZN staining.

Of the 25 isolates that were subtyped, 19 (76%) belonged to *C. hominis*, 5 (20%) were *C. parvum,* and 1 (4%) was *C. meleagridis*. These findings are consistent with results found in previous studies in Africa and other developing countries, where 79-90% of infections are due to *C. hominis* (Xiao and Feng, 2008b). Likewise in South Africa, mainly *C. hominis* and a small proportion of *C. parvum* were detected in two studies (Leav et al., 2002b; Samie et al., 2006). The predominance of *C. hominis* has also been observed in other pediatric populations in Africa, such as in Malawi (Peng et al., 2003b), Kenya (Gatei et al., 2006a), and Uganda (Tumwine et al., 2005).

Four common *C. hominis* subtype families (Ia, Ib, Id and Ie) are usually observed in humans in developing countries (Xiao, 2010). These findings are in accordance with a previous study of HIV-positive children in South Africa, where a high diversity of *C. hominis* was observed (Leav et al., 2002b); however in the latter study, the subtype family Ie was not detected, while If, a subtype family rarely reported in other countries, was found. In the present study: the most common *C. hominis* subtype family observed was If, followed by Ie, Id and Ia, and Ib (Table 2). The *C. hominis* Ie subtype identified, IeA11G3T3, has been reported in human infections from other developing countries (Xiao, 2010), such as Nigeria (Akinbo et al., 2010).

The *C. hominis* subtype family Id has previously been widely reported in other countries in Africa (Kenya, Malawi and South Africa), India, and Latin America (Peru) (Leav et al., 2002a; Peng et al., 2003b; Strong et al., 2000; Xiao, 2010). Among the four Id subtypes identified in this study, subtype IdA24 was previously reported in Kenyan children (Gatei et al., 2006a). Both *C. hominis* Ib subtypes identified in this study, IbA9G3 and IbA10G2, are commonly found in humans in developing countries (Gatei et al., 2007; Peng et al., 2003b).

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In this study as well as in previous studies conducted in South Africa (Leav et al., 2002b) and in other developing countries (Xiao, 2010) the anthroponotic IIc subtype was the dominant *C. parvum* parasite. However, some other unusual anthroponotic *C. parvum* subtype families, such as IIe and IIb, have occasionally been described in India (Muthusamy et al., 2006),Malawi (Peng et al., 2003a) and Uganda (Akiyoshi et al., 2006). This is consistent with the findings in our study, where three different *C. parvum* subtype families were identified: IIc, IIb and IIe, with IIc being the most common subtype family (Table 2). These results suggest that *C. parvum* infection in children with diarrhoea in South Africa is likely to be of anthroponotic nature.

To our knowledge this is the first time that *C. meleagridis* has been detected and subtyped in South Africa. *Cryptosporidium meleagridis,* originally a possible mammalian *Cryptosporidium* species (Xiao et al., 2002), has been widely reported in avian hosts (Qi et al., 2011). This may explain the ability of *C. meleagridis* to infect a wide range of mammals, including humans, rodents and calves (Xiao et al., 2002). In several developing countries, a high prevalence of *C. meleagridis* has been reported in both immunosuppressed and immunocompetent humans (Xiao, 2010). Therefore, it has been recognized as an important human pathogen in Africa (Kenya), Latin America (Peru) and Asia (Thailand) (Gatei et al., 2006a; Hung et al., 2007; Xiao et al., 2001) and evidence of zoonotic transmission has been reported in a recent study on an organic Swedish farm (Silverlås et al., 2012). This study highlights the presence of *Cryptosporidium* in our sample of diarrhoeic children; however diarrhoea in our study population could also have been caused by other concomitant pathogens, such as *Escherichia coli* or Rotavirus. Due to the absence of asymptomatic control subjects, it is difficult to document the real importance of the presence of *Cryptosporidium* as a diarrhoea causing agent in children in South Africa. This study also extends our knowledge of the genetic diversity of *Cryptosporidium* species among diarrheic children in several regions of the country, and reports for the first time the presence of *C. meleagridis*, various subtypes of *C. parvum*, and the subtype family Ie of *C. hominis*. The presence of anthroponotic *C. parvum* subtype families and the predominance of *C. hominis* found in this study suggest that cryptosporidiosis in humans in South Africa is may be predominantly of anthroponotic nature. Nevertheless, studies in developing countries specifically targeting rural areas, where conditions of poverty and contacts with animals are

more common, may reveal a higher importance of zoonotic transmission in human cryptosporidiosis. Further molecular epidemiologic studies targeting populations over wider geographic areas are needed to better understand the distribution, epidemiology and public health impact of *Cryptosporidium* species and subtypes in southern Africa. In addition, the associated environmental risk factors, such as water sources and contact with domestic and wild animals, should be investigated further.

CHAPTER 3

THE PREVALENCE OF *CRYPTOSPORIDIUM* SPP. OOCYSTS IN WILD MAMMALS IN THE KRUGER NATIONAL PARK, SOUTH AFRICA

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ABSTRACT

This study determined the prevalence of *Cryptosporidium* spp. oocysts in faecal samples from elephant (*Loxodonta africana*), buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*) in the Kruger National Park (KNP) and an adjacent game reserve in South Africa. Two of the study areas were in close proximity to rural communities on the western KNP boundary and the third study area was located in the centre of the KNP. Fresh stool samples (n = 445) were collected and tested using an immunofluorescent antibody test (IFA) for *Cryptosporidium parvum*. A total of 278 of these were randomly selected (approximately 90 samples per wildlife species) and tested with the modified Ziehl Neelsen (MZN) staining technique for *Cryptosporidium* spp. The prevalence of C*ryptosporidium* spp. was highest in elephants (25.8% [95% confidence interval: 17.3, 35.9]), followed by buffalo (5.5% [1.8, 12.4]) and impala (4.3% [1.2, 10.5]). *Cryptosporidium parvum* showed similar patterns, being most prevalent in elephants (4.2% [1.5, 8.8]), compared to buffalo (1.4% [0.2, 5.1]) and impala (1.9% [0.4, 5.3]).

Twenty-nine samples, including MZN positive samples and IFA positive samples were retested using a real time PCR (rtPCR) technique. Of the 28 MZN-positive samples, 14 (50%) were positive with rtPCR and of the 9 IFA-positive samples 6 (67%) were confirmed positive by rtPCR. The prevalence of *Cryptosporidium* oocysts was significantly higher in both of the two study areas adjacent to the western KNP boundary compared to the area in the centre of the KNP (*OR* = 3.2 [1.2, 9.0]; *P* = 0.024). Our study demonstrates for the first time the presence of *Cryptosporidium* spp. in wildlife in South Africa. The transmission of this parasite between wildlife, domestic animals and humans is a plausible hypothesis and represents a potential risk for immunodeficient human populations.

INTRODUCTION

Cryptosporidium is a coccidian protozoan parasite that causes gastrointestinal disease in a wide range of wild and domestic animals and humans (Appelbee et al., 2005). In humans, severe, life-threatening diarrhoea occurs in neonates and immunocompromised individuals, especially in HIV positive patients, while in immunocompetent individuals the disease is self-

limiting or subclinical (Hunter and Thompson, 2005). *Cryptosporidium* oocysts can be transmitted directly between different host species via the faecal-oral route, or indirectly via contaminated food or water. A single oocyst is sufficient to produce infection and disease in a susceptible host (Ramirez et al., 2004). There are 16 recognized *Cryptosporidium* species and more than 33 genotypes based on molecular structure (Smith et al., 2007) which infect different animals. For example, *Cryptosporidium parvum* has been described in cattle, humans and other mammals (Abe et al., 2006; Graczyk et al., 2001; Ryan et al., 2003), *C. andersoni* in cattle (Ryan, 2003), *C. muris* in rodents, *C. felis* in cats (Fayer et al., 2006), *C. canis* in dogs (Xiao et al., 2002; Xiao et al., 2004; Zhou et al., 2004), *C. meleagridis* and *C. baileyi* in birds (Morgan et al., 2001) and *C. hominis* in humans (Xiao et al., 2004). Seven species (*C. hominis, C. parvum, C. meleagridis, C. baileyi, C. felis, C. suis and C. muris*) have been shown to be associated with human infection. Of these, *C. parvum* is the most commonly reported species of zoonotic importance, affecting humans and a wide range of domestic and wild animal hosts. The other species have been reported primarily in immunocompromised patients (Fayer et al., 2000; Gatei et al., 2002).

In South Africa, a *Cryptosporidium* spp. infection prevalence of 18% was reported in school children and hospital patients in the Venda region, close to the KNP (Samie et al., 2006). Wild animals from the KNP have been observed to commonly cross the park boundaries and it is not unusual to find them in communal lands adjacent to the park, where they are likely to come into contact or share the same grazing areas with domestic ruminants and humans (Jori et al., 2011). Wildlife may be an important contributor to the pool of this parasite within the environment, since several *Cryptosporidium* species/genotypes have been described in wild animal species (Appelbee et al., 2005; Gómez-Couso et al., 2007; Ramirez et al., 2004). In Tanzania, a study conducted in Mikumi National Park reported *Cryptosporidium* oocysts in African buffalo (*Syncerus caffer*), zebras (*Equus zebra*) and wildebeest (*Connochaetes gnou*) with an overall prevalence of 25% (Mtambo et al., 1997). The prevalence of *Cryptosporidium* has been also studied in African primates and was found to be 53.7% in vervet monkeys (*Chlorocebus pygerythrus*) and olive baboons (*Papio anubis*) in Kenya (Muriuki et al., 1997). However, there is a dearth of information on the occurrence of *Cryptosporidium* spp. in wild animals in southern Africa, despite the socio-economic importance of wildlife in that region (Jori et al., 2011).

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Laboratory diagnosis is based on microscopic identification of *Cryptosporidium* oocysts (conventional acid-fast staining), immunological methods (monoclonal antibody techniques) in faecal smears or on more recently developed molecular techniques (Samie et al., 2006). Microscopic analysis of stained faecal smears is the most widely used screening method for *Cryptosporidium*. It has been reported that the sensitivity of the MZN acid fast technique for the detection of *Cryptosporidium* oocysts in faecal smears was 79.3% in cattle and 67.7% in pigs, while the specificity was reported as being 100% in both species (Quílez et al., 1996a). Molecular analysis such as polymerase chain reaction (PCR) has allowed a better understanding of the taxonomy, the zoonotic potential of these variants and the epidemiology of the disease (Appelbee et al., 2005).

The objective of this study was to investigate the prevalence of *Cryptosporidium* spp. oocysts in three common wildlife species in the KNP, South Africa: elephant (*Loxodonta africana*), buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*). In addition, we investigated whether the prevalence in wildlife populations in close proximity to humans and livestock differed from that in a more remote population with little or no contact with humans or livestock.

MATERIALS& METHODS

STUDY AREA

The study was conducted in the KNP and an adjacent private game reserve located in the province of Mpumalanga, South Africa. Three study areas were chosen: two within the KNP (Skukuza and Tshokwane), and the third within an adjacent game reserve (Sabi Sand) which is not fenced off from the KNP (Fig.1). Two of the study areas (Skukuza and Sabi Sand) are within a distance of 500 m from the boundary fence which separates wildlife from communally grazed domestic animals. Two major rivers, the Sabie and the Sand, cross adjacent communal lands before entering the park within the two study areas (Skukuza and Sabi Sand). The third study area (Tshokwane) is located in the centre of the KNP with little exposure to human settlements, livestock or major rivers draining from populated areas.

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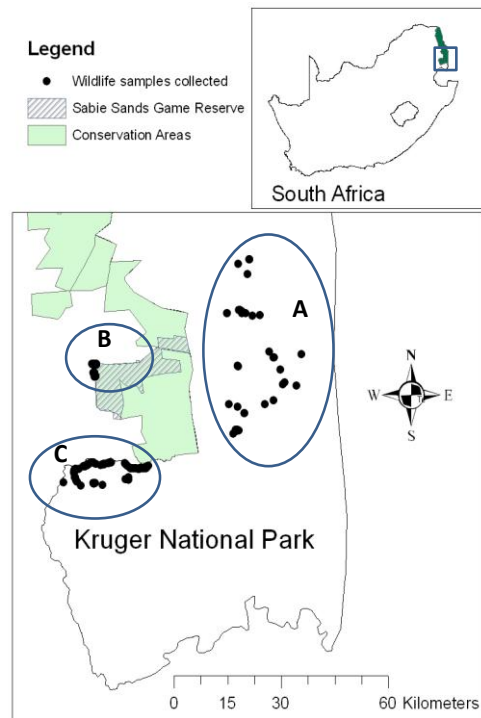
Figure 2: Study areas for collection of faecal samples from wildlife species: A (Tshokwane); B (Sabi Sand) and C (Skukuza).

FAECAL SAMPLING

During the dry season, in May/June 2008, fresh faeces from buffalo, elephant and impala were collected from the three study sites in the KNP and the Sabi Sand Game Reserve. Herds or single animals were selected in each study area according to convenience, i.e. proximity to a road and observed defecation by the animals. The samples were collected where the animals had just been grazing and the faeces were visibly fresh. Samples were considered fresh when the surface of the dropping was still moist. A total of 445 samples were collected: 141 from buffalo, 144 from elephant and 161 from impala. In each study area, between 40 and 60 samples were collected from each animal species and a maximum of 10 samples were taken from animals in the same herd. To avoid sampling the same animal a game guard estimated the movement of animal herds and sampling took place accordingly. The age and health status of the sampled individuals could not be assessed.

LABORATORY ANALYSIS

The faecal samples were transported to the laboratory on ice, where they were diluted with potassium dichromate (2.5%) and then stored at 4°C. All 445 samples were analyzed using a commercially available immunofluorescent antibody (IFA) test kit (Waterborne Inc., New Orleans, USA). The IFA technique is a specific anti-*Cryptosporidium parvum* monoclonal antibody technique and was used for detecting oocysts from different *Cryptosporidium* species. The faeces were diluted in phosphate buffered saline and filtered through a double layer of gauze. Twenty five ml of the filtered dilution was pipetted and placed in the well of an immunoflourescent slide (2-well slides) and processed according to the manufacturer's instructions. Briefly, the well was fixed with methanol and stained with the monoclonal antibody reagent. For each sample, the entire well was examined by epifluorescence microscopy at 65× magnification (Fluorescence microscope, Zeiss Axiovert 200, Germany). Criteria for a positive test result included fluorescence, as well as size and shape of the oocyst. A buffalo faecal sample with high numbers of *C. parvum* oocysts was included in each batch of samples as a positive control.

From the 445 faecal samples, 278 (approximately 30 from each species in each study area) were randomly selected to be analyzed with the MZN staining technique for detecting *Cryptosporidium* spp. oocysts. Before the staining process, the oocysts were concentrated by the formalin ether sedimentation technique (Casemore, 1991). The stained slides were then observed under the microscope with oil immersion using the 50× objective and the 100× objective was used to observe the internal structure of the oocyst. *Cryptosporidum* oocysts appeared as pink to red spherical structures.

All samples that tested positive either with MZN or with IFA were then tested using a real time PCR (rtPCR) protocol. DNA was isolated from preserved stool samples after 7 cycles of freeze and thaw, using the QIAamp Stool mini kit (Valencia, CA, USA). A rtPCR ~~method~~ protocol designed to detect most *Cryptosporidium* spp. was used for the detection of *Cryptosporidium* as previously described (Samie et al., 2006).

STATISTICAL ANALYSIS

Data were entered into a spreadsheet and then analyzed using Epi-Info (Version 3.3.2., CDC Atlanta, 2005) and Stata 11.1 (StataCorp, College Station, TX, U.S.A.). The prevalence of *Cryptosporidium* oocysts (MZN-positive and IFA-positive), was calculated by species and study area, with 95% exact binomial confidence limits. Prevalences were compared between species and between study areas using multiple logistic regression. The significance of the interaction between species and study area was also tested. The fit of the logistic regression models was tested using the Hosmer-Lemeshow goodness-of-fit test. The level of significance used was α = 0.05.

RESULTS

The prevalence of *Cryptosporidium* spp., using the MZN technique, by species and by study area, with 95% confidence limits, is shown in Table 3. The prevalence of *C. parvum*, determined using IFA, by species and by study area, with 95% confidence limits, is shown in Table 4.

Table 3: Prevalence of *Cryptosporidium* spp. oocysts in faecal samples from wildlife species in Kruger National Park, South Africa, determined using the modified Ziehl-Neelsen staining technique, with 95% confidence intervals.

	Skukuza			Sabi Sand			Tshokwane			Total/species		
	Positives	%	95% CI	Positives	%	95% CI	Positives	%	95% CI	Positives	%	95% CI
Elephant	12/35	34.3%	[19, 52.2]	10/28	35.7%	[18.6, 55.9]	2/30	6.6%	[0.8, 22.1]	24/93	25.8%	[17.3,5.9]
Buffalo	1/31	3.2%	[0.07, 16.2]	2/29	6.9%	[0.8, 22.7]	2/30	6.6%	[0.8, 22.1]	5/91	5.5%	[1.8, 12.3]
Impala	1/31	3.2%	[0.08, 16.7]	2/34	5.9%	[0.7, 19.7]	1/29	3.5%	[0.08, 17.7]	4/94	4.2%	[1.2, 10.5]

Table 4: Prevalence of *Cryptosporidium* spp. oocysts in faecal samples from wildlife species in Kruger National Park, South Africa, determined using the direct immunofluorescent antibody test, with 95% confidence intervals.

	Skukuza			Sabi Sand			Tshokwane			Total/species		
	Positives	%	95% CI	Positives	%	95% CI	Positives	%	95% CI	Positives	%	95% CI
Elephant	4/50	8%	[2.2, 19.2]	0/44	0%	[0, 6.5]	2/50	4%	[0.48, 13.7]	6/144	4.2%	[1.5, 8.8]
Buffalo	1/50	2%	[0.05, 10.6]	1/40	2.5%	[0.06, 13.2]	0/50	0%	[0, 5.3]	2/140	1.4%	[0.17, 5.1]
Impala	0/52	0%	[0, 5.6]	3/59	5%	[1.1, 14.1]	0/50	0%	[0, 5.8]	3/161	1.8%	[0.38, 5.3]

A total of 29 samples, including 28 MZN-positive samples and 9 IFA-positive samples were tested using rtPCR. Of the 28 MZN-positive samples, 14 (50%) were positive with rtPCR and of the 9 IFA-positive samples, 6 (67%) were confirmed positive by rtPCR.

The prevalence of *Cryptosporidium* spp. varied between the three study areas; Tshokwane, in the middle of the KNP, had a lower prevalence than the two areas adjacent to the western boundary (Table 3). Prevalence was highest in elephants, compared to buffalo and impala, with the highest prevalence being found in elephants adjacent to the boundary fence. The effects of species and area on prevalence of *Cryptosporidium* spp., based on MZN results, are shown in the results of the logistic regression model in Table 5. The combined prevalence in the two study areas adjacent to the KNP boundary fence was significantly higher than in the Tshokwane area (*OR* = 3.2 [95% confidence interval 1.2, 9.0]; *P* = 0.024).

Table 5: Effect of wildlife species and study area on prevalence of *Cryptosporidium* spp. oocysts in faecal samples from wildlife species in Kruger National Park, South Africa

Variable	Level	Odds ratio	95% CI (*OR*)	*P*-value
Area	Tshokwane	1*	–	–
	Sabi Sand	3.6	1.2; 11.1	0.023
	Skukuza	2.9	1.0; 8.8	0.057
Species	Impala	1*	–	–
	Buffalo	1.3	0.3; 5.2	0.665
	Elephant	8.4	2.8; 25.8	<0.001

Hosmer-Lemeshow goodness-of-fit χ^2 (7 *df*] = 5.22; *P* = 0.633

* Reference level

Although the prevalence of *Cryptosporidium* spp. appeared to vary between species and study area, the logistic regression model (not shown) did not show any significant differences. There was no significant interaction between species and area in either of the models. Both models showed adequate fit.

DISCUSSION

The results of this study show that *Cryptosporidium* spp. infection is present in some of the most common large mammal species in the KNP. This is the first report of the circulation of *Cryptosporidium* spp. in southern African wildlife and one of the few reports in African wildlife. The highest prevalence was detected among the elephant population and the prevalence was significantly higher in the areas adjacent to the fence of the KNP in the Skukuza and Sabi Sand areas.

The main risk factor for livestock, humans and wildlife to contract *Cryptosporidium* spp. infection is known to be a contaminated environment (Mtambo et al., 1997). Therefore wildlife, especially elephants, exiting the KNP may be potentially important disseminators of *Cryptosporidium* oocysts within the environment, exposing domestic animals, humans to possible infection. However, it is equally possible that humans or livestock may be a source of infection for wildlife, which could explain the finding that wildlife living adjacent to the fences of the park had a higher prevalence of *Cryptosporidium* spp. infection. It is known that *Cryptosporidium* oocysts survive longer periods in a humid environment (Robertson et al., 1992) and are therefore likely to be more prevalent during the rainy season than during the dry season (Gonzalez-Moreno et al., 2013; Katsumata et al., 1998). The fact that our samples were collected during the dry season (May/June) may therefore explain the relatively low prevalence detected in our study and therefore further studies should be done during the rainy season to test this hypothesis of seasonality. However, other studies have shown conflicting results; a higher prevalence of cryptosporidiosis was found during the dry season in cattle and people in Kenya (Kange'the et al., 2012), and in cattle in one study area in Malawi (Banda et al., 2009). The sensitivity and specificity of MZN for detection of *Cryptosporidium* oocysts in faeces from wild animals have not been reported. A sensitivity of 79.3% and specificity of 100% for the MZN technique was described for the detection of *Cryptosporidium* oocysts in cattle (Muriuki et al., 1997). Also, low specificity and sensitivity have been described in humans – 40.6% and 52.0%, respectively (Arrowood and Sterling, 1989; Rickard et al., 1999), suggesting that the possibility of false positives in our study cannot be excluded.

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Using rtPCR, 50% of MZN-positive and 67% of IFA-positive samples were confirmed positive. The remaining MZN positive and IFA-positive samples which tested negative by rtPCR could possibly be false positives. However, PCR inhibitors such as bilirubin, bile salts or complex polysaccharides, which would reduce the sensitivity of rtPCR, may be present in wildlife faeces. In addition, since the rtPCR does not detect all known *Cryptosporidium* genotypes, it is possible that the wildlife samples may have contained genotypes that could not be detected by this method. Further research is necessary to characterise the genotypes circulating in wildlife and to validate diagnostic methods for the surveillance of *Cryptosporidium* in wildlife species.

This study shows for the first time that *Cryptosporidium* spp. are circulating in wildlife populations in the KNP. However, in order to determine the zoonotic importance of *Cryptosporidium* spp. at the wildlife/livestock/human interface and to establish possible links between *Cryptosporidium* spp. in humans, livestock and wildlife, further studies should be done on the prevalence and molecular characterisation of *Cryptosporidium* strains in domestic animals and human patients from communal areas adjacent to KNP.

CHAPTER 4

MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM* SPECIES AT THE WILDLIFE/LIVESTOCK INTERFACE OF THE KRUGER NATIONAL PARK, SOUTH AFRICA

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ABSTRACT

Molecular characterization of *Cryptosporidium* spp. was done on isolates from African elephant (*Loxodonta africana*), African buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*) and native domestic calves collected during May and June 2008 at the wildlife/livestock interface of the Kruger National Park (KNP), South Africa. A polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA gene was used in faeces from 51 calves (3-12 months of age), 71 buffalo, 71 impala and 72 elephant, and sequencing of the 18S rRNA gene was done on PCR-RFLP-positive wildlife samples. *Cryptosporidium* spp. were detected in 7.8% (4/51) of the calves and identified as *C. andersoni* (2/4) and *C. bovis* (2/4). Four of the 214 wildlife samples were positive for *Cryptosporidium* with a prevalence of 2.8% each in impala and buffalo. *Cryptosporidium ubiquitum* was detected in two impala and one buffalo, and *C. bovis* in one buffalo. A concurrent questionnaire conducted among 120 farmers in the study area investigated contacts between wildlife species and livestock. Buffalo and impala had the highest probability of contact with cattle outside the KNP. Despite the fairly low prevalence found in wildlife and cattle, the circulation of zoonotic *Cryptosporidium* spp., such as *C. ubiquitum*, should be investigated further, particularly in areas of high HIV infection prevalence. Further studies should target younger animals in which the prevalence is likely to be higher.

INTRODUCTION

Cryptosporidium spp., protozoan parasites of the phylum Apicomplexa, have a wide spectrum of hosts including humans, domestic animals and wild mammals, birds, reptiles, amphibians and fish (Xiao and Feng, 2008a). Cryptosporidiosis is a common cause of diarrhoea in humans and animals, but is usually self-limiting in the immunocompetent host. However, in young or immunosuppressed hosts, such as HIV-infected patients, the parasite can cause severe and life-threatening diarrhoea (Xiao et al., 1999). Humans can acquire *Cryptosporidium* infections via several transmission routes, such as direct contact with infected persons (person-to-person transmission), animals (zoonotic transmission), or contaminated fomites, or ingestion of contaminated food (foodborne transmission) or

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water (waterborne transmission) (Xiao, 2010). The role of animals in the transmission of human cryptosporidiosis is nevertheless not clear (Xiao and Feng, 2008a). This is largely due to the fact that traditional diagnostic tools do not have the ability to differentiate between human-pathogenic and non-human-pathogenic species. Recently, molecular tools have been developed to detect and differentiate *Cryptosporidium* spp. at species/genotype and subtype level (Caccio, 2005; Xiao and Ryan, 2004). These tools have contributed to a better understanding of the transmission of cryptosporidiosis in humans and animals (Xiao, 2010). In a recent survey in four hospitals from South Africa, *Cryptosporidium* was detected in 12.2% of children with diarrhea. However, most of the identified strains were found to be non-zoonotic (Abu Samra et al., 2012). At least 10 species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. suis*, *C. muris*, *C. andersoni,* and *C. ubiquitum*) have been associated with human disease, although *C. hominis* and *C. parvum* remain the most common species (Nichols et al., 2010; Xiao, 2010).

Cattle are considered to be an important source of zoonotic cryptosporidiosis, since they are regarded as a major host of *C. parvum*. However, cattle are also commonly infected with *C. andersoni*, *C. bovis,* and *C. ryanae* (previously called the deer-like genotype) and the occurrence of these different species is usually related to the age of the host: *C. parvum* is mostly found in pre-weaned calves, *C. ryanae* in post-weaned calves and *C. andersoni* in yearling and adult cattle (Santín and Fayer, 2007). However, *C. bovis* and *C. ryanae* have been detected in pre- and post-weaned calves and adult cattle of all ages, which suggests that the occurrence of these species may not be age-related (Feng et al., 2007b). A species similar to *C. bovis* was also detected in one adult yak (*Bos grunniens*) in China and genotyping revealed only three nucleotide mutations in the target gene (Feng et al., 2007b). Thus, cattle and yaks are the only animal species in which *C. bovis* has been reported.

Cryptosporidium spp. have commonly been reported in wild mammals worldwide. Using microscopy, *Cryptosporidium* oocysts have been detected in faeces of African buffalo (*Syncerus caffer*), zebra (*Equus quagga*) and wildebeest (*Connochaetes taurinus*) in Mikumi National Park, Tanzania (Mtambo et al., 1997), and more recently in African buffalo, impala (*Aepyceros melampus*) and elephant (*Loxodonta africana*) in KNP, South Africa (Abu Samra et al., 2011). Recent molecular studies have described 11 *Cryptosporidium* species and nearly 30 genotypes of unknown species status in wildlife (Feng, 2010). Some of the

Cryptosporidium spp. detected in wild or captive mammals include *C. parvum*, *C. muris*, *C. canis*, *C. meleagridis*, *C. bovis*, C. andersoni, and *C. hominis*. Genotyping studies have mostly been done in captive artiodactyls and the following *Cryptosporidium* spp. have been found: *C. parvum* in red deer (*Cervus elaphus*), fallow deer (*Dama dama*), addax (*Addax nasomaculatus*), Arabian oryx (*Oryx leucoryx*), gemsbok (*Oryx gazella*) and sable antelope (*Hippotragus niger*); *C. ubiquitum* in sika deer (*Cervus nippon*), blesbok (*Damaliscus pygargus phillipsi*), nyala (*Nyala angasii*) and ibex (*Capra sibirica*); and *C. andersoni* in European wisent (*Bison bonasus*) (Feng, 2010). The white-tailed deer (*Odocoileus virginianus*) is the only free-ranging artiodactyl in which genotyping has been done and *C. parvum*, *C. ubiquitum* and *C. ryanae* were the major species reported (Feng et al., 2007a; Perz and Le Blancq, 2001; Xiao et al., 2002).

Most of the above genotypes are host specific, having been found only in closely related host species. Studies in various regions of the world suggest a strong host-adaptation by these parasites and limited potential for cross-species transmission among different species. Therefore it was concluded that these host adapted species and genotypes of *Cryptosporidium* do not pose a major threat to public health (Feng et al., 2007a). However, an exception is *C. ubiquitum* which infects a wide range of hosts, including wild and domesticated ruminants, rodents, carnivores and primates including humans (Fayer et al., 2010). *Cryptosporidium ubiquitum* has a wide distribution, is a common species in sheep and the most common *Cryptosporidium* species found in storm runoff water, and is therefore likely also to be present in wild mammals (Jiang et al., 2005).

On the periphery of protected areas in Africa, abundant populations of wildlife cohabit with livestock and rural communities, with livestock and wildlife often sharing grazing and water sources. This wildlife/livestock/human interface is a suitable environment for the circulation of common pathogens between the three compartments. As is the case with other neglected zoonosis, studies of cryptosporidiosis in this context are scarce. In the case of the KNP interface, studies on zoonotic diseases are particularly important, since the prevalence of HIV/AIDS in rural communities in the area is amongst the highest in the world (Shisana et al., 2009). Therefore, the primary goal of this study was to detect and characterize *Cryptosporidium* spp. in three common African wildlife species (elephant, African buffalo and impala) and indigenous cattle at the wildlife/livestock/human interface of the KNP. A

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second objective was to estimate, using a questionnaire, the extent of contact between livestock and cattle in communities close to the KNP boundary, and thereby to assess the potential for cross-species transmission of *Cryptosporidium* spp.

MATERIALS& METHODS

STUDY AREA

The KNP covers nearly 20,000 km^2 of semi-arid savannah in the north-eastern Lowveld region of South Africa. It is bordered to the east by Mozambique and to the north by Zimbabwe. The western border adjoins communal grazing areas and private game reserves in Mpumalanga and Limpopo provinces (Figure 3).

This study area for cattle comprised twelve diptanks in the communal grazing lands, within 5 km from the KNP boundary, in the Bushbuckridge area, the most populated region adjacent to the KNP. In those communal lands, livestock owners bring their cattle every week to diptanks, managed by the communities, to be dipped in order to protect them against tick-borne diseases. The most common cattle breed in that area is the local Nguni type, used for meat and milk consumption.

Three study areas were chosen: two within the KNP (Skukuza and Tshokwane), and the third within Sabi Sand, which is not fenced off from the KNP (Figure 3). Two of the study areas (Skukuza and Sabi Sand) are within a distance of 500 m from the boundary fence which separates wildlife from communally grazed domestic animals. However, they differ substantially in terms of the interactions that can occur between wildlife and cattle. In the case of privately owned game reserves such as Sabi Sand, the 2.4 m high electric fence is generally well maintained, electricity is functional, and it has been reported that incidents of wildlife escaping the KNP are rare in those cases (Jori et al., 2011) and grazing cattle do not have the possibility of entering within the game reserve and interacting with wildlife.

On the contrary, the 2.4 m high electric fence adjacent to Skukuza in the KNP is only partially electrified and bordered by communal pastoral land. This allows the frequent movement of

different wildlife species, including buffalo and impala, into grazing areas outside the KNP and the incursion of cattle from communal rural areas into the KNP for grazing and drinking (Brahmbhatt et al., 2012; Jori et al., 2011). The third study area (Tshokwane) is located in the centre of the KNP with little exposure to human settlements or livestock and no known interactions between wildlife and cattle.
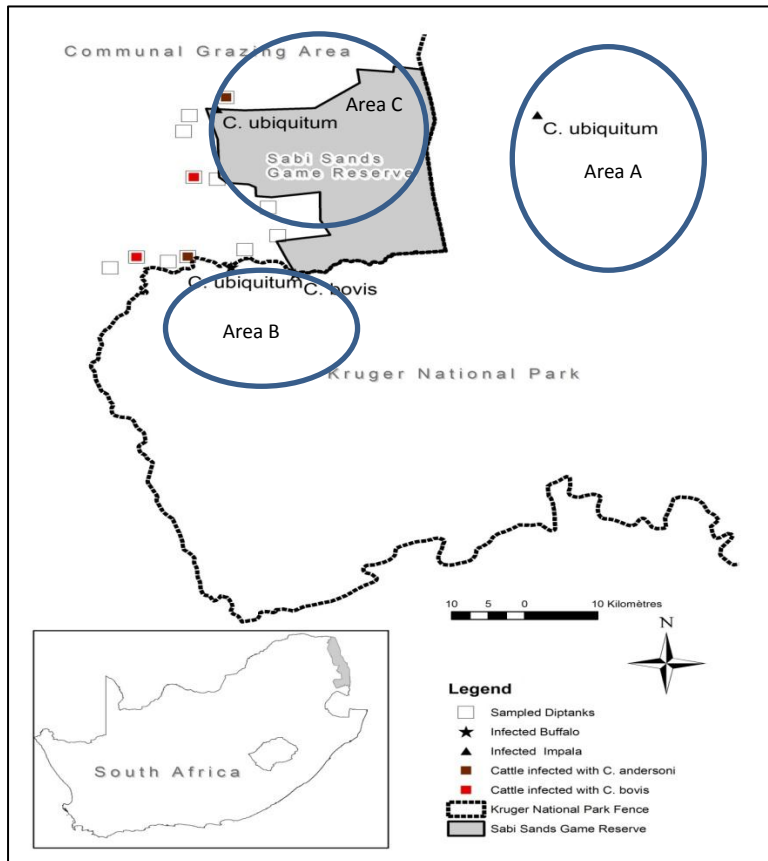
Figure 3: Map of the study area indicating the location of animals positive for *Cryptosporidium*.

ETHICS STATEMENT

The study protocol was approved by the Animal Use and Care Committee of the University of Pretoria (V014/08), and adhered to the guidelines of the South African National Standard (SANS 10386-2008): "The Care and Use of Animals for Scientific Purposes".

Before performing any work, the chiefs of the respective communities were contacted with the help of the Bushbuckridge state veterinarian, in order to seek their permission, inform them of the aims of the study and arrange meetings at the diptank to sample the cattle and implement the questionnaires. During these meetings the veterinary technicians managing the respective diptanks assisted in informing all cattle owners of the purpose of the study and that participation was voluntary. Confidentiality was assured, and all participants provided oral informed consent to participate in the questionnaire and for work to be conducted on their animals. Their consent was oral, as approved in the study protocol, due to the high proportion of illiterate people in the area. Consent was documented with the help of a local translator (veterinary technician at the respective diptanks) by giving a unique identification number to each participant, which further ensured protection of their privacy. The interview-based questionnaire was translated from English to the local language (Shangaan) with the help of veterinary technicians.

SAMPLING

A total of 214 fresh faecal samples were collected from free-ranging elephant (72), impala (71) and African buffalo (71) in the KNP and an adjacent game reserve, as previously described (Abu Samra et al., 2011).

All wildlife samples were collected from fresh droppings deposited in the field and were considered as fresh when the surface of the dropping was still moist. However, individual animals were not observed while defecating. Each collection area was visited only once, and in each study area between 40 and 60 samples were collected from each wildlife species (Abu Samra et al., 2011).

A total of 300 faecal samples were collected directly from the rectum of cattle at 12 diptanks located within the study area. Only samples of 51 calves ranging from 6 months to 1 year of age (the youngest age group within our sampled population), were analyzed during this study, due to the higher prevalence of *Cryptosporidium* in young cattle and water buffalo (*Bubalus bubalis*) compared to adult animals (El-Khodery and Osman, 2008; Xiao,

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2010). All wildlife and cattle samples were collected during May and June 2008 and were stored in 2.5% potassium dichromate at 4°C until processed.

QUESTIONNAIRE

A semi-structured questionnaire consisting of 15 questions was designed to gather information about cattle management, herd structure, grazing locations, and the occurrence, frequency and duration of observed contacts between cattle and common wildlife species in grazing areas both outside and within the KNP. The wildlife species considered were the most abundant large mammals in the KNP and included elephant, buffalo and impala. Contact was defined as the observation of cattle and wildlife species grazing together within an area the size of a football field (approximately 7000 m^2). The questionnaire was administered to 113 cattle owners who were chosen randomly at the various diptanks. The number of farmers who had observed wildlife in their livestock grazing fields during the previous year and the number of farmers who had observed contact between cattle and wildlife were each calculated as a proportion of the total number of respondents. The ratio between the proportion observing contact and the proportion observing wildlife, for each wildlife species, represented the probability that a farmer observing wildlife in the grazing area would also observe some contact between that wildlife species and cattle. This ratio was then used as a rough estimate of the probability that an escaped wild animal would associate with cattle. In order to assess the time that cattle and the different wildlife species remained together during those contacts, every farmer was asked to give an estimation of the duration of contact, choosing between three options: less than an hour, between 1 and 6 hours, or more than 6 hours. In order to assess the frequency of wildlife observations, every farmer was asked to give an estimation of the duration of contact, choosing between five options: 30 min to 1 h, 1 h to half a day, one day and one night, or more than one day and one night. In addition, the farmers were asked about the frequency of their cattle entering the KNP for grazing or drinking. The data were analyzed using Epi-Info (Version 3.3.2., CDC, Atlanta, 2005).

DNA EXTRACTION

DNA was extracted from 250 μl of each fecal samples (n=265) using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, Ohio). Prior to the extraction the samples were washed three times with deionized water, centrifuged and the pellet was transferred into a 2-ml tube containing lysing matrix E from the kit. After 1 ml of sodium phosphate buffer and 122 μl of MT buffer were added, the tubes were vortexed in a FastPrep instrument (MP Biomedicals, Solon, Ohio). The samples were processed further in accordance with the manufacturer's protocol.

CRYPTOSPORIDIUM GENOTYPING AT THE 18s rRNA LOCUS

An approximately 830-bp fragment of the 18s rRNA gene of *Cryptosporidium* was amplified by nested PCR as previously described (Feng et al., 2007a). Positive and negative controls were included in each analysis. The PCR products were examined by electrophoresis on 1.5% agarose gel and visualized after red dye staining. The positive PCR products were analyzed by restriction fragment length polymorphism (RFLP) analysis as described by Feng et al., (2007). The secondary PCR products were digested with the *SspI* and *MboII* enzymes and the *MboII* pattern identified *C. bovis* by having three visible bands at 412, 162 and 77 bp. DNA of *C. baileyi* was used as the positive control.

SEQUENCE ANALYSIS

The secondary PCR products of the wildlife samples were purified using Montage PCR filters (Millipore, Bedford, MA) and sequenced directly with secondary PCR primers using an ABI BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the manufacturer-suggested procedures. Sequences were read on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA) using the ChromasPro software (www.technelysium.com.au/ChromasPro.html). The nucleotide sequences obtained were aligned with reference *Cryptosporidium* sequences using ClustalX software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/).

RESULTS

CONTACT BETWEEN CATTLE AND WILDLIFE

The median cattle herd size of the farmers interviewed was 10 heads [Interquartile range: 7, 15.25], consisting of heifers (median = 5 [3;8]), bulls (median = 2 [1;4]) and calves (median = 2 [1;3]). A large majority of the cattle owners (98%) corralled their cattle at night.

Thirty-eight percent of the cattle owners reported that they had grazed their cattle on communal land within 500 m from the KNP fence and 36% (41/113) of farmers reported having observed some wildlife outside the KNP fence during the previous year. The species most commonly observed outside the KNP boundaries in the study area were buffalo (30/113; 27%), followed by elephant (25/113; 22%), and impala (24/113; 21%). Sixteen percent of the farmers (18/113) reported observations of contact between cattle and buffalo, 9.7% (11/113) observed contacts with impala and only one farmer reported contacts with elephant. The frequency of observation of the different wildlife species and cattle during the previous year is reported in Table 6. The probability of a farmer observing contact between livestock and a particular wildlife species, given that the species was observed outside the KNP fence, was therefore much higher for buffalo (Pr = 0.6) and impala (Pr = 0.46), than for elephant (Pr = 0.04). The average duration of contact reported by the different respondents is shown in Table 7. In addition, 6.2% of the interviewed farmers (7/113) reported taking their cattle inside the KNP for drinking.

Table 6: Frequency of observation of wildlife species by farmers during the previous year outside of the Kruger National Park

Frequency of observation	Species		
	Buffalo (n=26 farmers)	Impala (n= 21 farmers)	Elephant (n= 22 farmers)
Once a year	10.6	10.6	8.8
Twice a year	9.7	3.5	6.2
Once every 3 months	5.3	5.3	4.4
Once a month	-	2.7	1.8
Never	74.3	77.9	78.8
Total	**100**	**100**	**100**

Table 7: Duration of contact observed between cattle and wildlife species on the boundary of the Kruger National Park, as reported by cattle farmers.

Duration of contact	Species		
	Buffalo (n=18 farmers)	Impala (n=11 farmers)	Elephant (n=1 farmer)
0-60mns	56%	64%	100%
1-6 hours	17%	9%	-
>6 hours	28%	27%	-

CRYPTOSPORIDIUM GENOTYPING

The results of the genotyping are summarized in Table 8.

Table 8: *Cryptosporidium* species identified from impala, African buffalo and domestic calves at the wildlife/livestock interface of the Kruger National Park.

Animal Species	Number tested	*Cryptosporidium* positive (%)	*C. ubiquitum*	*C. bovis*	*C. andersoni*
African elephant	72	0	0	0	0
Impala	71	2 (2.8%)	2	0	0
African buffalo	71	2 (2.8%)	1	1	0
Domestic calves	51	4 (7.8%)	0	2	2

WILDLIFE

PCR amplification of the 18S rRNA gene was successful in 4/214 wildlife specimens (1.86%): 2/71 African buffalo (2.8%; 95% C.I. 0.3, 9.8%) and 2/71 impala (2.8%; 95% C.I. 0.3, 9.8%). All 72 elephant samples were PCR-negative (0%; 95% C.I. 0, 4.1%). The RFLP banding patterns indicated the presence of *C. bovis* and *C. ubiquitum*. Sequencing of the 18S rRNA gene PCR product confirmed the presence of these two *Cryptosporidium* species/genotypes: *C. ubiquitum* in one buffalo and two impala, and *C. bovis* in one buffalo. Three of the four positive samples (two *C. ubiquitum* and one *C. bovis*) were collected along the western boundary of the KNP and the adjacent Sabi Sand private game reserve, close to the adjoining communal grazing areas.

CATTLE

Four of the 51 cattle samples (7.8%; 95% C.I. 2.2, 18.9%) were positive for *Cryptosporidium* spp. using nested PCR for the 18S rRNA locus. Restriction analysis using the enzymes *SspI*

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and *MboII* showed banding patterns specific for *C. bovis* and *C. andersoni* in two different individuals each (Figure 4).

The infected animals originated from four different diptanks, all located in communal grazing areas within 5 km from the KNP boundary.
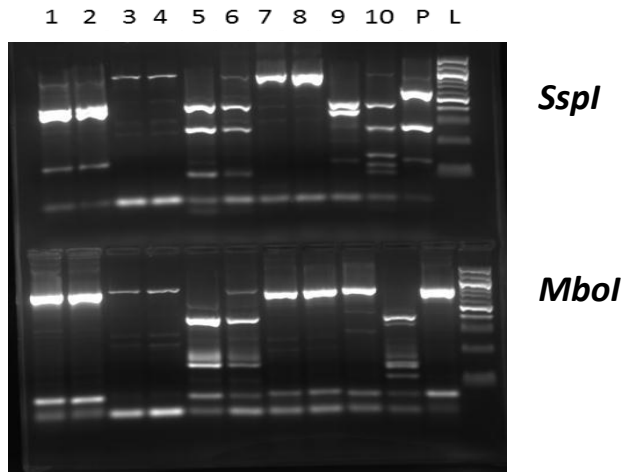
Figure 4: Differentiation of four common bovine *Cryptosporidium* species by *SspI* (upper panel) and *MboII* (lower panel) RFLP analysis. Lane L (ladder) 100 bp molecular markers; Lane P (positive control, *C. baileyi*); *C. andersoni* (lanes 1,2); negative (lanes 3,4), *C. bovis* (lanes 5,6); negative (lanes 7,8), *C. andersoni* (lane 9) and *C. bovis* (lane 10).

DISCUSSION

Despite the widespread occurrence of interactions between livestock, wildlife and human populations in sub Saharan Africa, little is known about the circulation of *Cryptosporidium* species in this specific epidemiological context. This could be of particular importance in rural communities in southern Africa, among whom HIV prevalence is high and exposure to zoonotic pathogens can be life threatening.

Molecular characterization of *Cryptosporidium* species and genotypes has contributed to a better understanding of the biological diversity of this parasite at species/genotype and subtype levels (Xiao and Feng, 2008a). To our knowledge, this study is the first one to apply molecular diagnostics to free-ranging African wildlife.

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Cryptosporidium ubiquitum was isolated from African buffalo and impala in this study. Apart from white-tailed deer in North America (Perz and Le Blancq, 2001), this is the first report of the presence of *C. ubiquitum* in any free-ranging wild ruminant species. Due to its lack of strong host specificity, *C. ubiquitum* has been detected in faeces from more animal species and over a greater geographic range than most species of *Cryptosporidium* (Feng, 2010). It has been found in a variety of wild rodents (Feng et al., 2007a), in water samples in various geographic locations, and in humans worldwide (Fayer et al., 2010). *Cryptosporidium ubiquitum* has also been found in faeces from wild, captive and domesticated ruminants including white-tailed deer (*Odocoileus virginianus*), blesbok (*Damaliscus dorcas phillipsi*), mouflon (*Ovis aries orientalis*), nyala (*Tragelaphus angasii*), sika deer (*Cervus nippon*) and domestic sheep. Due to its increasing occurrence in humans, *C. ubiquitum* has been described as a public health concern (Xiao and Feng, 2008a). A large proportion of the human cases were described in industrialized nations, where it ranked fourth amongst *Cryptosporidium* spp. infecting humans. More human cases have been ascribed to *C. ubiquitum* than to *C. canis* (Fayer et al., 2010). Very little is known about *C. ubiquitum* circulating in rural settings.

African buffalo and impala are two of the most common large wildlife species living in the KNP and in many other African game reserves. Recall bias could have played a role in the quantification of wildlife observations, which were estimated retrospectively for the previous year. It should be noted however, that a similar method to estimate wildlife observations along the KNP fence provided results that were biologically plausible (Jori et al., 2011). This is also the case for the results of our questionnaire, which confirm contacts between cattle and wildlife that are consistent with other studies (Brahmbhatt et al., 2012). Equally, our estimates of the probability of contact between livestock and the different wildlife species were higher for buffalo and impala than for elephant, which also makes biological sense. When those species graze in the same areas as cattle they seem to have a high probability of contact with cattle grazing on communal land. In addition, contacts may also occur by cattle entering the KNP for grazing and drinking, as seen in our study and reported elsewhere (Brahmbhatt et al., 2012; Jori et al., 2011). It is important to note that contact, as defined in our study, does not necessarily imply direct contact but includes indirect contact via shared water or grazing. Because of this sharing of grazing and water

between cattle and wildlife, the potential exists for transmission of *C. ubiquitum* and other pathogens between species at the wildlife/livestock/human interface (Jori et al., 2011).

Cryptosporidium bovis was found in one African buffalo sample in our study. To our knowledge, *C. bovis* is specific for cattle and, except for one reported isolation from a yak (*Bos grunniens*) in China (Feng et al., 2007a), has not been detected in other species. A possible explanation for our finding is that buffalo could have become infected from cattle either through direct contact or through the contamination of shared water sources or pasture. Another possibility is the existence of a closely related *C. bovis*-like genotype in buffalo; further studies of *Cryptosporidium* infected buffalo are necessary to confirm this.

The prevalence of *Cryptosporidium* spp. in faecal samples from wildlife reported here using molecular techniques was lower than that previously reported using microscopy in elephants, buffalo and impala (Abu Samra et al., 2011). In that study, observed prevalences in the three species using modified Ziehl-Neelsen (MZN) staining were 25.8%, 5.5% and 4.2% respectively, and using IFA staining for *Cryptosporidium* the prevalences were 4.2%, 1.4% and 1.8%, respectively. All positive elephant samples and several positive samples from other species tested negative in this study. The sensitivity and specificity of diagnostic tests for *Cryptosporidium* spp. have not been investigated in these wildlife species. Differences between the findings reported in this study and those observed by microscopy were higher in elephant than in impala and buffalo. A possible explanation is that the extremely fibrous consistency of elephant faeces or the presence of unknown, closely similar organisms or structures could have contributed to the detection of a higher number of false positives than for ruminant species such as buffalo and impala.

The low prevalence observed in wild bovids in this study was comparable to that generally observed in adult domestic cattle and water buffalo. Further studies in wildlife should target younger animals, which may be more likely to be infected.*Cryptosporidium bovis* and *C. andersoni* were the only species found in our sample of indigenous cattle. These two species, together with *Cryptosporidium parvum* and *C. ryanae* are the most common species reported in cattle worldwide (Xiao, 2010), of which *C. parvum* is the only species of zoonotic importance. C*ryptosporidium bovis* is considered to be of low zoonotic importance, although a novel *C. bovis* genotype has been described in a worker on a dairy farm in India (Khan et

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al., 2010). This study suggested that 'unusual' species may play a role in human infections, but since such findings are rare, their public health importance is considered limited (Khan et al., 2010).

Numerous molecular epidemiological studies of cryptosporidiosis have been conducted in dairy cattle in industrialized countries, but very few studies on *Cryptosporidium* species in native cattle have been reported from developing countries. Molecular characterization of *Cryptosporidium* spp. in native breeds of cattle in Nigeria and in Zambia demonstrated the absence of *C. parvum* in young indigenous cattle. The study in Nigeria reported the presence of *C. bovis* (7.2%), *C. ryanae* (4.1%), and *C. andersoni* (2.5%) in native cattle and suggested that, due to the absence of zoonotic *C. parvum* in young cattle, *Cryptosporidium* spp. may not be of zoonotic importance in that area (Maikai et al., 2011). A similar pattern was described in Zambia, where *C. parvum* was identified in isolates from commercial farms but not in traditionally reared calves (Geurden et al., 2006). The results of our study are therefore consistent with the findings reported from native calves in Nigeria and Zambia. However, the absence of *C. parvum* may also be attributed to the fact that this study did not target pre-weaned animals. Age-related occurrence of *Cryptosporidium* spp. in cattle has been reported in several studies (Ng et al., 2011; Santín and Fayer, 2007; Xiao, 2010). In order to investigate the possible occurrence of *C. parvum* in native calves in Mpumalanga province, South Africa, further molecular studies specifically targeting pre-weaned calves should be conducted.

This study suggests that the prevalence of *Cryptosporidium* in adult wildlife in the KNP is low; however, one of the circulating species (*C. ubiquitum*) is potentially of major zoonotic importance. The *Cryptosporidium* species found thus far in cattle at the wildlife/livestock interface are of minor zoonotic importance. However, the results of our questionnaire show that contacts between wildlife and cattle occur, particularly through cattle entering the park for drinking and grazing and the isolation of *C. bovis* from buffalo suggests that transmission of *Cryptosporidium* spp. could happen between wildlife and cattle. People in rural communities living close to the KNP, many of whom may be immunosuppressed due to HIV infection, can potentially become infected through direct contact with wildlife (e.g., by hunting) or their environment (e.g., contaminated water sources). Further studies on *Cryptosporidium*, targeting young domestic and wild animals and children at the

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wildlife/livestock/human interface are necessary in order to understand the circulation of different *Cryptosporidium* species and their zoonotic potential.

CHAPTER 5

PREVALENCE, GENOTYPES AND RISK FACTORS OF *CRYPTOSPORIDIUM* INFECTION IN CHILDREN AND CALVES AT THE LIVESTOCK AND HUMAN INTERFACE OF THE KRUGER NATIONAL PARK, SOUTH AFRICA

Manuscript in preparation

ABSTRACT

This study investigated the prevalence and the species/genotype distribution of *Cryptosporidium* among children (<5 years) and calves (<6 months) living in a communal farming area adjacent to the Kruger National Park (KNP), South Africa. The modified Ziehl-Neelsen (MZN) staining technique was applied for initial screening; *Cryptosporidium* oocysts were detected in 8/143 stool samples of children and in 2/352 faecal samples of calves. All MZN-positive samples from children were analysed by 18S rRNA-PCR and revealed a prevalence of 5.6% (8/143); positive samples were amplified at the GP60 gene and further subtyped by GP60 sequence analysis. Clear sequences were obtained from 4 isolates: *C. hominis* (3/4) and *C. meleagridis* (1/4). Randomly selected samples (n=36) from calves 0-4 months of age were analysed by 18S rRNA-PCR and 11 tested positive (30.6%; 95% CI: 16.3%, 48.1%), suggesting that the true prevalence of infection was much higher than indicated by the MZN test. 18S sequencing revealed the presence of *C. bovis* and *C. ryanae* in two cattle samples. The detection of cattle-specific *Cryptosporidium* spp. in calves and the presence of only anthroponotic species in children suggest that the public health concern of the parasite as a zoonosis in this region may be low.

INTRODUCTION

Cryptosporidium is a coccidian parasite of the phylum Apicomplexa and infects a wide range of animals, including humans. Cryptosporidiosis is associated with mild to severe diarrhoea which is typically self-limiting in immunocompetent hosts but can become chronic and life-threatening in immunocompromised and young individuals (Ramirez et al., 2004). Because of the wide host range of *Cryptosporidium* spp., the zoonotic importance of cryptosporidiosis has been recognised for some time (Xiao and Feng, 2008a). Nevertheless, the role of animals, both wild and domestic, in the transmission of human cryptosporidiosis has not been completely understood. Recent developments in molecular biological techniques allow detection and identification of *Cryptosporidium* spp. at species/genotype and subtype levels, contributing to better comprehension of the epidemiological patterns of cryptosporidiosis. Thus far more than 20 *Cryptosporidium* species have been established, in

addition to more than 50 *Cryptosporidium* genotypes that do not have any species status (Plutzer and Karanis, 2009).

Humans are most commonly infected by two species of *Cryptosporidium*: *C. parvum* which infects primarily cattle in addition to humans and may be of either zoonotic or anthroponotic origin, and *C. hominis* which infects primarily humans. *Cryptosporidium meleagridis*, *C. felis* and *C. canis* are responsible for a small number of cryptosporidiosis cases in humans, and have mainly been reported in immunosuppressed patients (Xiao and Feng, 2008a); however, *C. meleagridis* infection rates were reported to be fairly high (9/109) in diarrhoeic children from Peru (Cama et al., 2008b). Geographic differences in the incidence of *C. hominis* and *C. parvum* in humans have been described; in European countries both *C. parvum* and *C. hominis* are equally common in humans, in the Middle East *C. parvum* has predominantly been described, while in developing countries, *C. hominis* is responsible for 70-90% of infections (Xiao, 2010; Xiao and Feng, 2008a). Not all *C. parvum* subtypes in humans result from zoonotic infection; a significant proportion of human *C. parvum* isolates have not been found in animals (IIc, IIb, IIe) and may therefore not have originated from a bovine reservoir. *Cryptosporidium parvum* infection in humans in developing countries appears to be mostly of anthroponotic origin; for example in Lima, Peru and Jamaica *C. parvum* subtype IIc was the only subtype family detected in humans, while in India, Malawi, Uganda, Kenya and South Africa subtype families IIb and IIe were seen in addition to subtype family IIc (Xiao, 2010).

Four *Cryptosporidium* species are known to infect cattle: *C. parvum*, *C. bovis*, *C. andersoni* and *C. ryanae*, and their occurrence is known to be age-related. *Cryptosporidium parvum* has been detected mainly in pre-weaned calves (<2 months), *C. bovis* and *C. ryanae* in weaned calves, and *C. andersoni* in yearlings and adult cattle (Fayer et al., 2000; Santín et al., 2004; Xiao, 2010). Overall, prevalence of *Cryptosporidium* spp. is reported to be highest in pre-weaned calves, followed by weaned calves and adult cattle (Santín et al., 2004). However, studies conducted in native cattle in Nigeria and Zambia have failed to detect *C. parvum* in young indigenous cattle (Geurden et al., 2006; Maikai et al., 2011).

In South Africa, cryptosporidiosis has been reported in children in various locations; one study was conducted in a hospital in Durban, KwaZulu-Natal, where a prevalence of 24.8% in

diarrhoeic children was found (Leav et al., 2002b), and in a more recent study an overall prevalence of 12.2% has been reported in hospitalised diarrhoeic children from four different provinces in South Africa (Gauteng, Mpumalanga, North West Province and KwaZulu-Natal) (Abu Samra et al., 2013b). Thus far, these have been the only studies in South Africa in which the genotypes and subtypes of *Cryptosporidium* spp. have been determined. The predominant species in both studies was *C. hominis* with four different subtype families (Ia, Ib, Id, Ie and If), followed by *C. parvum* of anthroponotic origin (IIc, IIe and IIb), while the latter study additionally described the presence of *C. meleagridis* in one patient. Another study (Samie et al., 2006) reported a prevalence of cryptosporidiosis of 18% in school children and hospitalized patients in Limpopo Province, South Africa, and confirmed that *C. hominis* was more common than *C. parvum*. Data on cryptosporidiosis in cattle in South Africa are sparse; a prevalence of 33.64% has been reported in adult cattle from the southern Free State (Bakheit et al., 2008) and more recently in one study conducted at the wildlife/livestock interface area of the KNP; a prevalence of 8% was reported in weaned calves and genotyping revealed the presence of *C. bovis* and *C. andersoni* (Abu Samra et al., 2013a). However, no investigations on *Cryptosporidium* spp. circulating simultaneously in young children and animals living at the wildlife/livestock/human interface have been done.

Risk factors associated with *Cryptosporidium* infection in cattle have been identified by several authors. In Egypt younger age, wet season, poor hygiene and drinking from wells or underground water were significantly associated with the risk of cryptosporidiosis in buffalo calves (El-Khodery and Osman, 2008) (El-Khodery, 2008). In Zambia there was a significantly higher prevalence in intensively raised dairy calves compared to extensively raised ranch beef calves and traditionally reared calves (Geurden et al., 2006). In Kenya, Kange`the et al. (2012) carried out a participatory risk assessment to estimate the risk to humans of zoonotic *Cryptosporidium* in dairy farms and identified infection from milk, vegetables and contaminated water as the main exposure pathway.

In the present study we analysed the diversity of *Cryptosporidium* at species and subtype levels in young children and calves at the wildlife/livestock/human interface along the boundary of the KNP. The objective of this study was to estimate prevalence of infection, to

identify links between human and cattle infections and to identify potential risk factors associated with cryptosporidiosis in the two compartments.

MATERIALS& METHODS

STUDY SITE

The study took place in a communal farming area, the Mnisi Traditional Authority (MTA), situated in a rural environment in the north-eastern Lowveld area of Mpumalanga Province, South Africa. This study area is situated adjacent to private protected wildlife areas, continuous with the KNP (Figure 5).
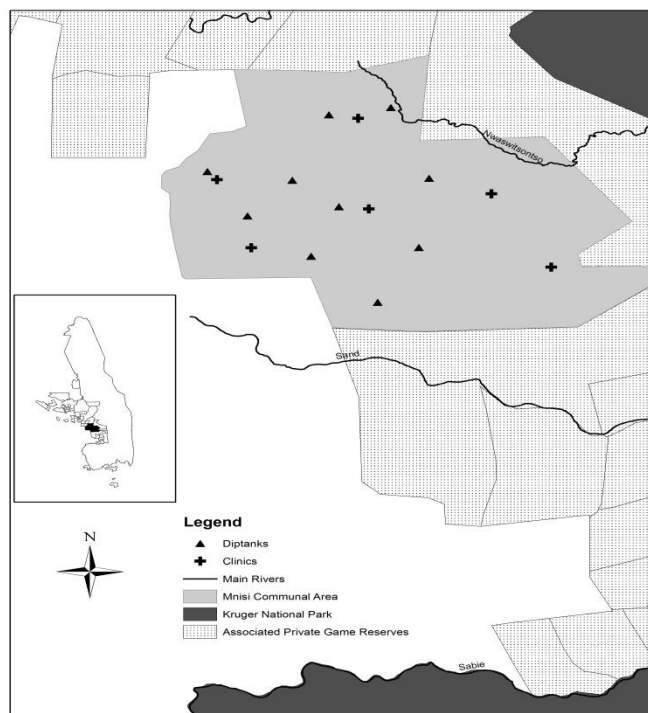
Figure 5: Study area for the location of clinics and diptanks where sampling took place

In this area, subsistence farmers rely mostly on cattle farming, the most common breed being the indigenous Nguni, used for milk and meat consumption. Cattle diptanks have been

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established throughout the region, and every cattle herd is taken for dipping and inspection once a week. The MTA community hosts a population of over 40,000 people and eight health centres are located within this area. The prevalence of HIV infection in this community has been reported to be as high as 30% (van Rooyen, personnel communication, 2012).

ETHICS STATEMENT

This study was approved by the Animal Use and Care Committee of the University of Pretoria for animal sampling and by the Research Ethics Committee of the University of Pretoria for human sampling (Protocol no. 5/2011). Before initiating the work, all stakeholders were contacted in order to seek their permission; animal health technicians and a local field assistant assisted in informing all cattle owners of the purpose of this study and that participation was voluntary. Nurses at participating health care centres informed their patients of the purpose of the study and if parents agreed to their children's participation a consent form was signed.

SAMPLING COLLECTION

Children

From March to June 2012, during the end of the rainy season and the start of the dry season, stool samples from 143 children <5 years old were collected at six different rural clinics in the study area, regardless of reported symptoms of diarrhoea. Stool samples were collected with parental consent and on a volunteer basis in collaboration with nurses working at the clinic. The parent of each participant were asked to complete a brief questionnaire to gather information about age, contacts with domestic animals, source of drinking water, whether water was boiled before drinking, and history of diarrhoea since birth, including frequency and length of diarrhoeic episodes.

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Calves

During the same period, faecal samples were collected from pre-weaned calves (0-6 months of age) at 11 different dip tanks in the study area. A sample size was calculated to estimate a 5% prevalence with 2.5% allowable error, using the formula $1.96^2 PQ/L^2$ (Thrusfield, 2005), where P is the estimated prevalence (0.05), $Q = 1–P$ and L is the allowable error (0.025), given a required minimum sample size of 292; ultimately, 352 calves were sampled. Faecal samples were collected directly from the rectum of calves and each dip tank was visited only once to avoid repeated sampling of individual calves. A brief questionnaire was completed by interviewing the herdsman accompanying each sampled animal. Closed questions (dichotomous and multiple choice) for ascertaining age, herd size and source of drinking water were used. The body condition score (based on the 1-4 scale) and consistency of faeces (solid/soft/watery) were documented by research assistants.

All faecal samples from children and calves were transported on ice to the National Institute for Communicable Diseases (NICD) in Johannesburg, where they were diluted with 2.5% potassium dichromate and stored at 4°C until processed.

LABORATORY ANALYSIS

At the NICD faecal samples from calves and children were concentrated by the formalin-ethyl acetate sedimentation method and thin smears were stained using the MZN technique. The stained slides were examined by light microscopy with oil immersion using the 50x and 100x objectives to detect *Cryptosporidium* spp. oocysts. Due to limited resources only MZN positive faecal samples of children (n = 8) were sent to the Istituto Superiore di Sanità, Rome, the European Reference Laboratory for Parasitology, for molecular analysis. Of all calf samples only animals within the age group 0-4 months were selected and 36 samples of this age category were randomly selected for molecular analysis.

DNA EXTRACTION

Prior to DNA extraction, *Cryptosporidium* oocysts were purified from faeces by a caesium chloride (CsCl) gradient. Purified oocysts were suspended in 25 µl of buffer (10 mM Tris pH 8.3, 50 mM KCl containing 0.5% w/v Tween 20). After freeze-thawing (15 cycles), samples were heated for 15 min at 100°C and then centrifuged for 2 min at 13 000 rpm to remove particulate matter. Finally, 2-5 µl of supernatant was used for PCR amplification (Bonnin et al., 1996) (Bonnin et al., 1996).

CRYPTOSPORIDIUM AMPLIFICATION AND GENOTYPING AT THE 18s rRNA LOCUS

The extracted DNA of samples from children (n=8) and calves (n=36) were amplified at the 18S rRNA gene using a nested PCR assay (Tiangtip and Jongwutiwes, 2002). The amplified 18S rRNA gene of *Cryptosporidium* was analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under a UV transilluminator. PCR products were purified and sequenced on both strands. Sequences were assembled using the software SeqMan version 7.1 (DNAStar) and compared with those available in GenBank using BLAST (Pruitt et al., 2005).

CRYPTOSPORIDIUM AMPLIFICATION AND SEQUENCING OF THE GP60 GENE

The extracted DNA of all samples identified as *C. hominis* were then amplified at the GP60 gene on a Veriti 96 thermocycler (Applied Biosystems), using external primers as described by Strong et al. (2000) and internal primers as described by Drumo et al. (Drumo et al., 2012). PCR products were purified using spin columns and sequenced on both strands. Sequences were assembled using SeqMan version 7.1 (DNAStar). A BLAST search against the GenBank database was used to identify *C. hominis* GP60 genotypes.

STATISTAL ANALYSIS

Data were entered into a spreadsheet and then analysed using Epi-Info (Version 3.3.2., CDC Atlanta, 2005) and Stata 12.1 (StataCorp, College Station, TX, U.S.A.). The prevalence of *Cryptosporidium* spp., based on the 18S PCR, was calculated for calves and for children with 95% exact binominal confidence limits. The univariable association between each potential risk factor and *Cryptosporidium* infection was assessed separately for calves and for children using Fisher's exact test. In addition, all factors were entered into multivariable exact logistic regression models to adjust for confounding. Statistical significance was assessed at *P*< 0.05.

RESULTS

CHILDREN

Results of *Cryptosporidium* testing in faecal samples from 143 children and univariable associations with potential risk factors from 141 questionnaires (two interviews were not completed) are shown in Table 9. Details on diarrhoeic episodes were not consistently reported and therefore only history of diarrhoea was considered during the analysis. Eight (4.9%; 95% CI: 1.6%, 8.9%) faecal samples were found positive by the MZN staining method and were confirmed positive by PCR. Therefore the prevalence of *Cryptosporidium* infection in children was estimated to be 5.6% (95% CI: 2.4%, 10.7%). At least one positive sample was detected in five out of the six clinics surveyed. The univariable associations of the five potential risk factors with the presence of *Cryptosporidium* in children were not significant, and there was no significant association with a history of diarrhoea (Table 9); likewise, the exact logistic regression model revealed no significant associations between any of the factors and *Cryptosporidium* infection (Table 10).

Four of the eight 18S PCR-positive samples were successfully sequenced and *C. hominis* (3/4) and *C. meleagridis* (1/4) were identified. The unclear sequences were characterized by overlapping nucleotides along part of the sequence, possibly originating from a mixture of *Cryptosporidium* species in a particular sample, or by difficulties in sequencing AT-rich

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tracts. PCR amplification of the GP60 gene and subtyping at GP60 locus was done on *C. hominis* and two subtype families were identified, namely Ib (IbA12G3R2 and IbA10G2) and Ie (IeA11G3T3).

Table 9: Univariable association of factors with *Cryptosporidium* infection in 143 children <5 years old living in a communal farming area at the wildlife/livestock/human interface

Variable	Level	Number of children	Prevalence of *Cryptosporidium* infection (%)	*P*-value
Age	0-2	15	6.7	0.765
	2-6	26	3.8	
	6-12	50	8.0	
	12-26	50	4.0	
Source of drinking water	tap	136	5.9	1.000
	other (fountain or river)	7	0.0	
Boil water before drinking	yes	24	4.2	1.000
	no	119	5.9	
Contact with cattle	yes	33	6.1	1.000
	no	108	5.5	
Contact with other animals (cat, dog, goat)	yes	105	5.7	1.000
	no	36	5.5	
History of diarrhoea	yes	84	3.6	0.437
	no	56	7.1	

Table 10: Potential risk factors associated with *Cryptosporidium* infection in 143 children <5 years old: results of a multivariable exact logistic regression model

Variable	Level	Odds ratio	95% CI (OR)	*P*-value
Age	0-2 months	1*	-	-
	<6 months	0.62	0.01, 55.7	1.000
	6-12 months	0.89	0.07, 51.2	1.000
	>12 months	0.34	0.01, 26.7	0.880
Contact with cattle	No	1*	-	-
	Yes	0.87	0.07, 7.10	1.000
Contact with other animals (cat, dog, goat)	No	1*	-	-
	Yes	1.31	0.17, 15.5	1.000
Source of drinking water	other	1*	-	-
	tap	0.08	0.002, ∞	1.000
Boiling water before drinking	No	1*	-	-
	Yes	1.09	0.01, 20.3	1.000

*Reference level

CALVES

Only 2/352 calf samples (0.6%; 95% CI: 0.1%, 2.0%) were positive by MZN. Of the 36 calves <4 months of age that were randomly selected for 18S rRNA nested PCR, 11/36 were positive, giving an estimated prevalence of 30.6% (95% CI: 16.3%, 48.1%). These 11 PCR positives did not include the two MZN positives. At least one positive calf was detected in eight of the nine dip tanks sampled. None of the univariable associations of the three potential risk factors with the presence of *Cryptosporidium* in calves were significant (Table

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11); likewise, the exact logistic regression model revealed no significant associations with *Cryptosporidium* infection (Table 12).

Table 11: Univariable association of factors with the presence of *Cryptosporidium* in 36 calves <4 months old living in a communal farming area at the wildlife/livestock/human interface

Variable	Level	Number of animals	Prevalence of *Cryptosporidium* infection (%)	P-value
Age of animals	<1	10	30.0	0.498
	1-2	4	0.0	
	2-4	22	36.3	
Herd size	1-10	17	29.4	0.426
	11-25	16	25.0	
	>25	3	66.7	
Source of drinking water	Dam	35	31.4	1.000
	River	1	0.0	

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Table 12: Potential risk factors associated with *Cryptosporidium* infection in 36 calves <4 months old: results of a multivariable exact logistic regression model

Variable	Level	Odds ratio	95% CI (OR)	P-value
Age	<1 month	1*	-	-
	1-2 months	0.87	0; 12.7	0.925
	2-4 months	1.40	0.2; 10.6	0.983
Herd size	1-10	1*	-	-
	11-25	0.99	0.14; 6.16	1.000
	>25	3.48	0.15; 235.0	0.669

*Reference level

Sequencing of the 18S rRNA gene PCR product revealed the presence of *C. bovis* and *C. ryanae* in two samples. Sequences of the remaining samples were not clear, but were characterized by overlapping nucleotides along part of the sequence.

DISCUSSION

In sub-Saharan Africa, cryptosporidiosis is recognized as an important disease in children and immunosuppressed adults (Mor and Tzipori, 2008). Most studies in sub-Saharan Africa have targeted diarrhoeic children, reporting various prevalences: 20% in Uganda (Akiyoshi et al., 2006), 18% in Zambia (Nchito et al., 1998), 9% in Tanzania (Cegielski et al., 1999) and 5.9% in Malawi (Morse et al., 2007). In South Africa, prevalences of 24.8% (Leav et al., 2002b) and 12.2% (Abu Samra et al., 2013b) have been reported in hospitalized diarrhoeic children. The most recent study involved a hospital located in close proximity to our study area (approximately 20 km southward), where 8.6% of diarrhoeic children were infected (Abu Samra et al., 2013b), compared to the 5.6% detected in the present study. From literature it is known that prevalence is higher in children with diarrhoea than in apparently

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healthy children from the same age group (Mor and Tzipori, 2008), which is in agreement with the findings of the present study. The present study targeted children <5 years of age, however their clinical status was not taken into consideration; non-diarrhoeic children may shed low concentrations of *Cryptosporidium* oocysts in faecal samples (DuPont et al., 1995) and due to the low sensitivity of the MZN technique (Balatbat et al., 1996; Ramirez et al., 2004) a large number of false negatives might have contributed to the lower apparent prevalence in children in this study. Elsewhere in Africa, comparable studies targeting calves of a similar age group have been conducted, using PCR as a diagnostic test; in Nigeria a prevalence of 16% was reported in native cattle less than one year of age (Maikai et al., 2011) and in Zambia 48% of dairy calves and 6.3% of traditionally-raised calves, all less than 3 months of age and within the same study area, were found to be infected with *Cryptosporidium* (Guerden et al., 2006). The PCR results of the present study are therefore broadly consistent with the findings in Nigeria and Zambia.

The presence of *C. bovis* and *C. ryanae* and the apparent absence of *C. parvum* in young calves in this study, are in agreement with findings from Nigeria (Maikai et al., 2011) and Zambia (Geurden et al., 2006). Both studies failed to demonstrate the presence of *C. parvum* in traditionally reared indigenous calves, indicating that native breeds of cattle may not be important in the transmission of zoonotic cryptosporidiosis.

The discrepancy in our results between the detection of *Cryptosporidium* from faeces of calves with the two diagnostic tests (MZN and PCR) is evident. PCR, due to its higher sensitivity, is more suitable for detecting *Cryptosporidium* infections in individuals with low parasite numbers, compared to MZN (Ramirez et al., 2004). In general, microscopic detection of *Cryptosporidium* oocysts in faecal samples suffers from low sensitivity as the detection limit of acid fast staining techniques, such as MZN, was reported to be 50 000 oocysts per gram of faeces (Balatbat et al., 1996). In comparison, PCR has a detection limit of up to 500 oocysts per gram of faecal sample (Paul et al., 2009; Xiao, 2010; Xiao and Feng, 2008a; Xiao et al., 1999). The present study did not target diarrhoeic calves and therefore the faecal samples analysed by MZN staining technique may have contained a low number of oocysts. Therefore poor sensitivity likely explains the high number of false negative results using the MZN technique in this study.

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The present study integrated the sampling of humans and livestock living within the same area. Few such studies have been carried out in sub-Saharan Africa. In Zambia, the occurrence of *Cryptosporidium* in farm workers, their household members and calves living on the same farms has been investigated. *Cryptosporidium* (mostly *C. parvum*) was detected in 6% of farm workers and 34% of calves and it was therefore suggested that zoonotic transmission may have occurred (Siwila et al., 2007). In Kenya, *Cryptosporidium* was detected during dry and wet seasons in dairy cattle (15% and 11% respectively), cattle-keeping families (4% and 0.3% respectively) and their non-cattle-keeping neighbours (5% and 0% respectively) and in HIV-positive people (5%) living in the same community (Kange'the et al., 2012). There were no significant differences in prevalence between the three groups of people and no overall association between *Cryptosporidium* infection in cattle and in people of the same households, suggesting that zoonotic cryptosporidiosis was not important in these communities. The findings of our study are comparable to the Kenyan study (Kange'the et al., 2012); both studies found a low prevalence of *Cryptosporidium* infection in humans, regardless of cattle ownership. In addition, we identified *Cryptosporidium* to the species and genotype level, confirming the predominance of anthroponotic *Cryptosporidium* (*C. hominis*) in children and of cattle specific genotypes (*C. bovis* and *C. ryanae*) in calves, supporting the conclusion that zoonotic transmission may not be important.

The predominance of *C. hominis* detected in children in this study is in accordance with the findings of other studies conducted in developing countries (Ramirez et al., 2004; Xiao, 2010). *Cryptosporidium hominis* is responsible for most human cases of cryptosporidiosis in Africa (Akiyoshi et al., 2006; Gatei et al., 2007; Morse et al., 2007). In South Africa, (Leav et al., 2002b) described for the first time the predominance of *C. hominis* in HIV-infected children and a few years later (Samie et al., 2006) detected a high prevalence of *C. hominis* (82%) among school children and hospitalized patients. In a recent study, *C. hominis* (76%), *C. parvum* (20%)and one case of *C. meleagridis* were described in diarrheic children from four provinces in South Africa (Abu Samra et al., 2013b). In the present study one sample was likewise identified as *C. meleagridis*; however, no *C. parvum* was detected. Four *C. hominis* subtype families are most commonly observed in humans (Ia, Ib, Id and Ie) in developing countries (Xiao, 2010). The *C. hominis* subtype family most frequently observed

in this study was Ib (2/3) followed by Ie (1/3). Within the *C. hominis* subtype family Ib, IbA10G2 is commonly seen in South Africa, Peru and India (Cama et al., 2008b; Gatei et al., 2007; Leav et al., 2002b) and is identical to the one identified in this study. IeA11G3T3 is identical to *C. hominis* Ie subtype previously identified in South Afrcia (Abu Samra et al., 2013b) and also reported in human infections from other developing countries (Xiao, 2010), including Nigeria (Akinbo et al., 2010). However, the *C. hominis* subtype IbA12G3R2 described in our study has, to our knowledge, never previously been described in sub-Saharan Africa.

In this study potential risk factors for *Cryptosporidium* infections in children and calves, which covered aspects of sanitation and zoonotic transmission, were found statistically non-significant. The lack of association between animal contact and *Cryptosporidium* infection further suggests that zoonotic transmission may not be important in our study area. However, the small sample size and low prevalence of cryptosporidiosis likely limited the power of our statistical analysis and our ability to identify risk factors.

In conclusion, this study failed to demonstrate the presence of zoonotic *Cryptosporidium* species in cattle and detected mainly anthroponotic species in children. These findings may suggest that zoonotic cryptosporidiosis in this study area is not a major public health concern. However, the prevalence of HIV/AIDS in many such rural communities is high and cryptosporidiosis is still considered the leading opportunistic disease in immunosuppressed people (Putignani and Menichella, 2010). Therefore, larger and more comprehensive studies, investigating more potential risk factors and including the role of waterborne transmission, are needed to better understand the epidemiology of *Cryptosporidium* spp. at the livestock and human interface.

CHAPTER 6

GENERAL DISCUSSION

In this study a low prevalence of cryptosporidiosis was detected in children, calves and three wildlife species at the KNP interface. Genotyping revealed the presence of predominantly anthroponotic *Cryptosporidium* species in children and the cattle-specific species in calves. Nevertheless, one zoonotic *Cryptosporidium* species was isolated in buffalo and impala. Movements of wildlife outside the park and crossing of cattle inside the KNP suggested that contact between wildlife and cattle exists. The occurrence of these contacts, combined with poor hygienic practices amongst the rural communities outside the KNP, suggest a potential for zoonotic transmission at this interface.

PREVALENCE OF *CRYPTOSPORIDIUM* INFECTION IN WILDLIFE, CATTLE AND HUMANS

Cryptosporidiosis is one of the most common causes of diarrhoea in humans and livestock (Santín et al., 2004) and is usually self-limiting in immunocompetent individuals but debilitating, persistent and even mortal in immunocompromised or young individuals (Ramirez et al., 2004; Santín et al., 2004). *Cryptosporidium* spp are commonly found in wild mammals worldwide, but most infections were reported in asymptomatic animals (Appelbee et al., 2005; Feng, 2010).

In sub-Saharan Africa, prevalences of cryptosporidiosis in humans (Akiyoshi et al., 2006; Areeshi et al., 2008; Cegielski et al., 1999; Gatei et al., 2006b; Kange'the et al., 2012; Morse et al., 2007; Nchito et al., 1998; Salyer et al., 2012) and cattle (Geurden et al., 2006; Kang'ethe et al., 2012; Nizeyi et al., 2002; Salyer et al., 2012; Swai et al., 2007) have been frequently reported, while very few studies on cryptosporidiosis in African wildlife (Mtambo et al., 1997; Salyer et al., 2012) have been carried out.

PREVALENCE IN WILDLIFE

The prevalence of C*ryptosporidium* in the present study was low but demonstrated the presence of some *Cryptosporidium* oocysts in three species of wild mammals: Impala, African buffalo and African elephant. However *Cryptosporidium* species/genotypes were only identified in impala and buffalo.

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The prevalence of infected animals was significantly higher in areas adjacent to the western boundaries of the KNP compared to areas in the centre of the park. Overall, the distribution of *Cryptosporidium*-positive wildlife within the KNP suggests that, if transmission between compartments does occur, humans or livestock may act as a source of infection for wildlife rather than the other way around. In addition, several rivers enter the KNP following a flow that goes from the outside to the interior of the KNP and which could act as a source of water-borne pathogens from anthropized to natural areas. To confirm this hypothesis, it would be necessary to investigate the presence of *Cryptosporidium* in water from rivers passing from communal land into the KNP. Nevertheless, considering the low prevalences found in wildlife, it is unlikely that these are a reservoir of infection for livestock and humans in our study area.

Little is known on the prevalence of cryptosporidiosis in wildlife in other African countries. Only one comparable study was conducted in Tanzania, where oocysts were detected in 22% (8/36) of African buffaloes, 28% (7/25) of zebras and 27% (7/26) of wildebeest (Mtambo et al., 1997). In that study, faecal samples were analysed by MZN and all positives were confirmed with an anti-*Cryptosporidium* monoclonal antibody test kit; however, no genotyping was conducted and therefore no identification of *Cryptosporidium* species was performed.

During the present study, *Cryptosporidium* species/genotypes were not identified in elephant samples, although some had tested positive using MZN and IFA. The fact that PCR, the most sensitive and specific diagnostic test of all three laboratory techniques applied during this study, did not detect any *Cryptosporidium* spp. in our elephant faeces may suggest that elephants in the KNP were not infected. However, various factors may have contributed to the failure to molecularly detect the parasite in elephants, such as small sample size, poor quality of samples, fitness of the primer sets and the presence of PCR inhibitors, commonly reported in ruminants (Thornton and Passen, 2004). No conclusion can therefore be made regarding the possible presence of *Cryptosporidium* infection in elephants in the KNP.

Concerning the results of buffalo and impala, low prevalence was detected by all three diagnostic techniques and the presence of two *Cryptosporidium* species (*C. ubiquitum* and *C.*

bovis) in two impala and one buffalo demonstrates for the first time that some wild ruminants in the KNP are infected.

While the presence of cryptosporidiosis has been described previously in impala and buffalo, they may be comparable to other studies conducted on *Cryptosporidum* spp. in wild ruminants (Feng et al., 2007a; Mtambo et al., 1997; Perz and Le Blancq, 2001; Rickard et al., 1999), no such comparison is possible for elephants as in this thesis cryptosporidiosis has been studied for the first time in that species.

PREVALENCE IN CATTLE

A number of prevalence studies in cattle in sub-Saharan Africa have been undertaken and results of these vary widely depending on the age group targeted and the husbandry system sampled. In Kenya, cryptosporidiosis was found in 20% of adult cattle from smallholder farms (Kang'ethe et al., 2012). In Uganda 38% of pre-weaned calves from dairy farms were found infected (Nizeyi et al., 2002) and 2.2% of traditionally reared adult cattle (Salyer et al., 2012). In Zambia cryptosporidiosis was found 48%, 8% and 6.3% of pre-weaned calves from dairy, beef and traditional husbandry systems respectively (Geurden et al., 2006). In Tanzania *Cryptosporidium* spp. infection was found in 19.7% of dairy cattle of all age from smallholder farms (Swai et al., 2007).

Abundant literature data indicated that more pre-weaned calves than post-weaned calves are infected with *Cryptosporidium*(Brook et al., 2008; El-Khodery and Osman, 2008; Khan et al., 2010; Maikai et al., 2011; Santín et al., 2004) and that prevalences are higher in intensive compared to extensive farming systems (Geurden et al., 2006).

Infection rates for post-weaned calves (7.8%) found in the present study are higher compared to the prevalence detected in traditionally reared cattle (2.2%) from Uganda (Salyer et al., 2012) , but lower than prevalence from smallholder farms in Kenya (20%) (Kang'ethe et al., 2012) and Tanzania (19.7%) (Swai et al., 2007).

The prevalence detected for pre-weaned calves (30.5%) are similar to the findings from Uganda (38%), despite the fact that the latter study targeted dairy farms, and higher

compared to the 6.3% of prevalence detected in traditional husbandry systems in Zambia (Geurden et al., 2006).

During the present research, a significantly higher prevalence of infection was detected in pre-weaned than in post-weaned calves (p = 0.009). However, the comparison of these two age groups might be biased by the fact that sampling took place in different study areas and during different years and seasons: Pre-weaned calves were sampled at the end of the rainy season and sampling of post-weaned calves happened at the beginning of the dry season. Therefore, seasonality may have accounted for these differences in prevalence. In order to draw any conclusions on age related differences in prevalence in calves, sampling of pre- and post-weaned calves should be organised within the same area and during the same period.

PREVALENCE IN HUMANS

Cryptosporidium infection prevalence in children varies considerably in sub-Saharan Africa, and is reported to be highest when associated with HIV infection and malnutrition, and when patients with diarrhoea are sampled (Mor and Tzipori, 2008). Among children with diarrhoea reported prevalence varies between 13.0% in Tanzania (Cegielski et al., 1999) and 73.6% in Uganda (Akiyoshi et al., 2006), 7.5% in Liberia (Højlyng et al., 1986) and 12.5% in Guinea-Bissau (Carstensen et al., 1987). In comparison, the prevalence in children without considering clinical symptoms has been reported in Kenya to be 4% and 5% during dry season in cattle-keeping and non-cattle keeping households, respectively (Kange'the et al., 2012), 5.9% in Malawi (Morse et al., 2007), 11.6% in Nigeria (Ayinmode et al., 2012) and 7.4% in Guinea-Bissau (Mølbak et al., 1993).

In the present study, prevalence of *Cryptosporidium* infection detected in diarrhoeic children from different parts of South Africa during the pilot study was higher (12.2%) compared to prevalence detected in apparently healthy children (5.9%) living at the wildlife/livestock/human interface. These results suggest a higher prevalence in diarrhoeic than in non-diarrhoeic children and are therefore comparable to prevalences described in diarrhoeic children in Tanzania (Cegielski et al., 1999) and Guinea-Bissau (Carstensen et al.,

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1987) and to the prevalences reported in non-diarrhoeic children in Kenya (Kange'the et al., 2012) and Malawi (Morse et al., 2007).

The prevalence of HIV infection in 2010 in Mpumalanga Province (within our study area) was reported to be 35.1%, the second highest prevalence of all provinces in the country (Shisana et al., 2009) and South Africa has one of the highest prevalences of HIV infection in the world (Shisana et al., 2009) Since the introduction of highly active retroviral therapy, the incidence of cryptosporidiosis has been reduced worldwide (Pozio et al., 1997); nevertheless, cryptosporidiosis is still a leading opportunistic infection in HIV infected people and should not be underestimated in epidemiological tracing and clinical follow up (Putignani and Menichella, 2010). During this study the HIV status of the participating children was not taken into consideration as this information is highly sensitive in South Africa and difficult to obtain. However, knowledge of the HIV status of sampled subjects would be valuable in future studies in order to determine whether cryptosporidiosis is a potential threat to human health in HIV-infected people in the study area.

DIAGNOSTIC TESTS

Diagnostic tests for the detection of *Cryptosporidium* infection in humans and livestock have often been described in literature. The modified Ziehl-Neelsen (MZN) staining technique is considered the gold standard test (Potters and Van Esbroeck, 2010) for diagnosis in humans, although it is a labour intensive technique and requires interpretation by well-trained microscopists. A sensitivity and specificity of 83.8% and 98.9%, respectively, have been reported in humans (Morgan et al., 1998), while in animals, lower sensitivity and specificity have been described for cattle and pigs (Quílez et al., 1996a). However, limitations in detecting *Cryptosporidium* with MZN in low oocyst shedding individuals have been acknowledged; The threshold for detection has been reported to be 10 000 oocysts per gramme of diarrhoeic stool and 500 000 oocysts per gram of non diarrhoeic stool (Balatbat et al., 1996; Chalmers et al., 2011; Jex et al., 2008). As a result, Balatbat et al (1996) suggested that more sensitive methods were needed to detect *Cryptosporidium* in asymptomatic or chronic carriers. Rapid immunoassays (enzyme immunoassays and fluorescent antibody assays), designed for simple diagnostic testing with minimal training,

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are commercially available. These tests are more expensive than conventional staining techniques and are therefore not commonly used as routine diagnostic methods. However, due to their higher sensitivity and specificity compared to MZN, they are suitable to detect *Cryptosporidium* infections in individuals with low parasite numbers (Ramirez et al., 2004). Their sensitivity and specificity have been evaluated in human faecal (Garcia and Shimizu, 1997) and some livestock faecal samples (Quílez et al., 1996a) but information on their performance in wild animal species is scarce.

Molecular techniques such as PCR provide a much higher sensitivity and specificity for the detection of *Cryptosporidium* in animals and humans compared to conventional microscopy. In addition, they ensure specific diagnosis up to species level (Paul et al., 2009; Sulaiman et al., 1999; Xiao, 2010).

DIAGNOSTIC TESTS IN WILDLIFE

It is important to be aware of the limitations of the currently available diagnostic tests for the detection of *Cryptosporidium* infection in wildlife species.

To our knowledge the sensitivity and specificity of diagnostic tests for detection of *Cryptosporidium* infection have never been reported in wildlife faecal samples. During this study samples from wild animals were analysed using three different diagnostic tests: the MZN staining technique, a commercially available IFA test kit (Chapter 3), and nested PCR/RFLP (Chapter 4). Combining the results from the different chapters, considerable variation between tests is evident; the MZN staining technique detected the highest number of *Cryptosporidium* oocysts in wild animals (Chapter 3), with highest apparent prevalence found in elephants, followed by buffalo and impala (Table 1). Lower prevalences were detected using the IFA test kit, with the highest prevalence in elephants, compared to buffalo and impala while PCR failed to detect *Cryptosporidium* species in elephants and revealed higher prevalence in buffalo and impala compared to IFA test kit (Table 13).

Table 13: Prevalence of *Cryptosporidium* infection in elephant, African buffalo and impala using three diagnostic tests

Test	Buffalo	Impala	Elephant
MZN	5.5%	4.3%	25.8%
IFA	1.4%	1.9%	4.2%
18S PCR	2.8%	2.8%	0%

The agreement between the MZN staining technique and the IFA test in the three wildlife species was quantified using the *kappa* statistic, which is a measure of the amount of observed agreement which is over and above that expected to occur by chance, and is scaled from 0 (no agreement) to 1 (perfect agreement) (Landis and Koch, 1977). The estimates of *kappa* (0.56) in buffalo (Table 14) and in impala (Table 15) indicate moderate agreement between the two tests in these species. However, the low prevalence and limited sample sizes result in a large degree of uncertainty in the estimates of kappa, reflected by the wide confidence intervals. In contrast, the estimate of *kappa* (0.26) in elephant (Table 16) indicates only fair to poor agreement (Thrusfield, 2005). In elephant faeces, MZN showed a much higher number of positives compared to IFA (Table 2) and to PCR (Table 1).

Table 14: Agreement between the modified Ziehl-Neelsen staining technique and the direct immunofluorescent antibody test for the detection of *Cryptosporidium* oocysts in buffalo faeces

Buffalo	MZN+	MZN-	Total
IFA+	2	0	2
IFA-	3	86	89
Tot	5	86	91

Test agreement: *kappa* = 0.56 (95% CI: 0.12, 0.99)

Table 15: Agreement between the modified Ziehl-Neelsen staining technique and the direct immunofluorescent antibody test for the detection of *Cryptosporidium* oocysts in impala faeces

Impala	MZN+	MZN-	Total
IFA+	2	1	3
IFA-	2	89	91
Tot	4	90	94

Test agreement: *kappa* = 0.55 (95% CI: 0.11, 1.00)

Table 16: Agreement between the modified Ziehl-Neelsen staining technique and the direct immunofluorescent antibody test for the detection of *Cryptosporidium* oocysts in elephant faeces

Elephant	MZN+	MZN-	Total
IFA+	5	1	6
IFA-	19	68	87
Total	24	69	93

Test agreement: *kappa* = 0.26 (95% CI: 0.05, 0.46)

The discrepancy between the results of the three diagnostic tests in elephants is very evident and may be explained by the following reasons:

First of all, the extremely fibrous elephant faeces may contain a variety of structures that can be confused with *Cryptosporidium* oocysts, such as fungal spores, fat globules and some bacterial spores (Casemore, 1991). Fungal spores are usually larger (6-10 µm) than *Cryptosporidium* oocysts (4-6 µm) (Casemore, 1991; Kara et al., 2011). The difference in size of ~~this~~ these structures is not extreme and might be confusing for an unexperienced examiner. General difficulties in the interpretation of *Cryptosporidium* oocysts by MZN microscopy has been acknowledged by several authors, both in human (Casemore, 1991; Chalmers et al., 2011) and cattle (Wee et al., 1996) faeces. Low specificity of the MZN technique has been reported previously mainly due to human errors in slide preparation and interpretation (Casemore, 1991; Wee et al., 1996). A combination of these above mentioned factors, might have contributed to the very low specificity and therefore high number of false positives in elephant, when using the MZN technique.

The IFA test is considered more sensitive than MZN in detecting *Cryptosporidium* oocysts, especially in human and animal faeces that contain few parasites and large amounts of debris (Casemore, 1991; Chalmers et al., 2011; Garcia and Shimizu, 1997; Quílez et al., 1996a). Even though the IFA technique applied in this study was a specific anti-*C. parvum*

monoclonal antibody technique, which is used for detecting *C. parvum* oocysts, cross-reactivity with other *Cryptosporidium* species has been reported (Teixeira et al., 2011). However, we cannot rule out the possibility that this diagnostic test may have failed to react with all *Cryptosporidium* spp. present in our samples which might explain the lower prevalence detected compared to MZN.

These given reasons may at least partly explain the discrepancy between MZN, IFA and PCR and the failure to detect *Cryptosporidium* in elephant samples using nested PCR. Nevertheless, PCR analysis is generally highly sensitive and specific and might therefore be considered a valid diagnostic test for the detection of *Cryptosporidium* in elephants, which would lead us to the conclusion that none or very low levels of *Cryptosporidium* spp. circulated in the sampled elephant population.

Finally, our results raise questions about the reliability of these diagnostic tests in all three wildlife species. For the moment, PCR may be considered the most reliable technique in detecting *Cryptosporidium* in wildlife species. However, its high cost limits its utilization to a large amount of samples (Paul et al., 2009). An alternative technique to use for future studies on wildlife samples may be the LAMP technique, which has previously described in cattle in South Africa (Bakheit et al., 2008). The LAMP assay provided a superior detection sensitivity than PCR, especially for screening of healthy animals (Bakheit et al., 2008) and is less cost effective.

DIAGNOSTIC TESTS IN CATTLE

All cattle samples were screened with the MZN technique, and in addition approximately 10% of these samples, all within the age group of 0-4 months, were retested by PCR. The discrepancy between the two test results (2/352 were positive with MZN and 11/36 with PCR) suggests a very low sensitivity of MZN technique, since a high number of apparently MZN-negative samples were confirmed positive by PCR. If considering samples detected as positive by PCR as true positives, our findings in cattle and humans confirm the low sensitivity and high specificity of MZN previously reported in other studies (Kara et al., 2011;

Morgan et al., 1998; Paul et al., 2009; Quílez et al., 1996a; Weber et al., 1991; Wee et al., 1996).

DIAGNOSTIC TESTS IN HUMANS

In the present study, human samples (children <5 years) from the wildlife/livestock/human interface were initially screened with the MZN technique and all MZN-positive samples (plus one suspicious sample) were confirmed positive by PCR. This suggests a high specificity of the MZN technique in human samples of this study. However, due to limited funding, only MZN-positives were retested by PCR. Therefore, the absence of PCR results in MZN negative samples did not allow estimation of the sensitivity of the test. Considering the low sensitivity of MZN reported by several authors (Casemore, 1991; Jex et al., 2008; Weber et al., 1991) the possibility of false negative results in the human samples cannot be excluded, especially when considering that apparently healthy children were targeted which, if infected, would likely have had low oocyst levels in their stool.

In conclusion, the sensitivity of MZN appears to be very low in human, cattle and wildlife samples of this study, while its specificity seems to be high in cattle and humans and very low in wildlife, especially in elephants. Therefore the detection of *Cryptosporidium* spp. in faecal samples from wildlife, cattle and humans may be more sensitively performed by PCR. However, in order to draw any more precise conclusions regarding test performance, further comparative evaluation of coprological diagnostic methods and PCR for detection of *Cryptosporidium* spp. is necessary, with special focus on wildlife species. For this purpose it will be necessary to obtain larger sample sizes from each species, preferably from at least two populations with different prevalence of infection, and to test each sample using multiple diagnostic tests. The data should then be analysed using methods for estimation of diagnostic sensitivity and specificity in the absence of a gold standard, such as Bayesian latent class analysis (Branscum et al., 2005).

SEASONALITY

SEASONALITY IN HUMANS

Peaks of *Cryptosporidium* infection during rainy seasons have been reported worldwide in humans. For example in Cost Rica (Mata et al., 1984), Kuwait (Sulaiman et al., 2005a), India (Das et al., 2006), Guinea Bissau (Mølbak et al., 1993), Zambia (Nchito et al., 1998) and Malawi (Morse et al., 2007) the prevalence of cryptosporidiosis was reported to be higher in children during rainy season. In Zambia, oocyst contamination of drinking water has been associated with higher prevalence of *Cryptosporidium* infection during the rainy season, possibly due to overflow of sewage during heavy rains and consequent contamination of surface water which might be used for drinking (Nchito et al., 1998).

During this study, children were sampled at the end of the rainy season. Previously, some authors have explained the lower prevalence of *Cryptosporidium* infections at the end of the rainy season (Mølbak et al., 1993), were explained by the development of immunity, after repeated exposure to *Cryptosporidium* oocysts with the initial rains (Mor and Tzipori, 2008). This hypothesis may suggest that low prevalence reported in children during this study, may be ascribed to the development of immunity. However, this assumption would need to be confirmed by additional studies, where sampling would be carried out during dry and rainy seasons and by the assessment of immunity in the patients.

SEASONALITY IN ANIMALS

In animals, seasonal effects on the level of cryptosporidiosis are less clear. For example, in Malawi seasonal variation in *Cryptosporidium* occurrence was reported in adult cattle and calves of two areas; one area had more infections during the rainy season, whereas another area a higher prevalence of infection was detected in the dry season (Banda et al., 2009). In Kenya, the prevalence in cattle was reported to be higher in the dry season than in the wet season (Kange'the et al., 2012). Possibly in these cases different confounding factors may

have played a role in the variation of *Cryptosporidium* infection, such as age of animals and husbandry system.

In the present study, the sampling periods were not planned to assess the effects of seasonality. Cattle sampling took place during two periods: May-June (beginning of the dry season) and March-May (end of the rainy season), however, neither sampling period was specifically representative of the dry or rainy season. Despite this lack of representativity, prevalence was lower during the first (dry season) than during the second (rainy season) collection period, with a prevalence of 7.8% and 31%, respectively (p = 0.009). Considering that sampling during both seasons did not take place within the same year and in the same study area, other confounding factors may also have influenced the prevalence.

Wildlife was sampled at the beginning of the dry season, when the decreasing survival period of oocysts in the environment could have contributed to the low infection rates detected. However, no rainy season samples were available for comparison.

The higher prevalence of *Cryptosporidium* infection during the rainy season may be explained by the fact that wet and humid weather conditions favour the survival of environmentally robust *Cryptosporidium* oocysts, which remain viable in water for over 140 days (Hooda et al., 2000). Moreover, storm water runoff during the rainy season contributes to environmental transport of faeces and therefore increased dissemination of oocysts (Caccio, 2005; Mor and Tzipori, 2008). The lower infection rate detected during the dry season may be due to higher temperatures and increased solar radiation which may inactivate *Cryptosporidium* oocysts in the environment (King et al., 2008). In addition, less water is available during this season, which limits the availability of infection sources.

Nevertheless, accurate assessment of the seasonality of cryptosporidiosis would require a more specific sampling design. Sampling of cattle, wildlife and children should preferably be organized during the peak of the rainy and the peak of the dry season within the same year, and other potential confounding factors such as age of study subjects and location should also be controlled.

ZOONOTIC IMPORTANCE OF CRYPTOSPORIDIOSIS

During this study, all PCR positive samples of diarrhoeic (Chapter 2) and apparently healthy (Chapter 5) children were genotyped. In both study populations the *Cryptosporidium* spp. detected were predominantly of anthroponotic origin, with the highest proportion being *C. hominis*, followed by *C. parvum* (of anthroponotic nature), and only one isolate in each study group was identified as *C. meleagridis*. *Cryptosporidium meleagridis* has been widely reported in avian hosts and in a wide range of mammals, including rodents, cattle and humans (Qi et al., 2011; Xiao et al., 2002). This species is regarded as an emerging human pathogen (Putignani and Menichella, 2010), being responsible, for example, for 1% of all infections in England (Caccio, 2005), but for 10-20% of infections in Peru and Thailand, where its prevalence is as high as that of *C. parvum* (Cama et al., 2008b; Gatei et al., 2002). Its occurrence in humans in our study area therefore raises the possibility that zoonotic transmission may occur, although *C. meleagridis* was not isolated from animals in this study.

All *Cryptosporidium* positive calves in our study were only infected with cattle specific *Cryptosporidium* spp., namely *C. bovis*, *C. ryanae* and *C. andersoni*.

Amongst the buffalo samples, one bovine species specific *Cryptosporidium* (*C. bovis*) was detected; however, one non-species specific *Cryptosporidium* (*C. ubiquitum*) was found in one buffalo and two impala. *Cryptosporidium ubiquitum* has been described in a large variety of animals, both wild and domestic, as well as in humans, but this is the first time that it is reported in African wildlife. Due to its wide distribution and its zoonotic potential its presence in wildlife in the KNP should be monitored further.

In this study, using molecular typing, we therefore found two *Cryptosporidium* species of zoonotic importance: *C. ubiquitum* in wildlife and *C. meleagridis* in both diarrhoeic and apparently healthy children. However, since none of these two species were detected simultaneously in animals and humans of this study, no direct evidence of zoonotic transmission was found. Finally the lack of links between animals and humans and the low prevalence of cryptosporidiosis detected in our study population suggests that the zoonotic importance of *Cryptosporidium* infection in our study area is limited, at least during the periods and in the areas where sampling took place.

POTENTIAL SOURCES OF INFECTION AND ROUTES OF TRANSMISSION AT THE WILDLIFE/LIVESTOCK/HUMAN INTERFACE

In developing countries sporadic cases, rather than outbreaks, of cryptosporidiosis have been reported and these mostly among HIV infected and diarrhoeic patients (Putignani and Menichella, 2010). In these countries, *Cryptosporidium* infections have been related to unhygienic living conditions, the use of pit latrines, improper disposal of wastewater and the use of surface waters as a major source of potable water (Putignani and Menichella, 2010). For example in Cameroon and Kenya, *Cryptosporidium* oocysts were detected in waste and surface waters, indicating that water can be contaminated by animal or human faeces, exposing users to zoonotic or anthroponotic species of *Cryptosporidium* (Gideon et al., 2007; Muchiri et al., 2009). *Cryptosporidium* infection has also been associated with contaminated fruit and vegetables, for example in Costa Rica where most vegetables and fruit from local agriculture markets were contaminated with *Cryptosporidium* spp. (Calvo et al., 2004). Possible source of contamination of such products may have been due to contaminated irrigation water or washing fruit and vegetables with infected water (Putignani and Menichella, 2010). Furthermore, contact with contaminated faeces transmitted by coprophagous transport hosts, such as birds and insects, has been identified as a potential risk factor for cryptosporidiosis (Smith et al., 2007). However, overall, livestock faecal pollution of water sources appears to be the leading cause for both outbreaks and sporadic cases of cryptosporidiosis in developing countries (Putignani and Menichella, 2010).

In South Africa, to date, no outbreaks of cryptosporidiosis due to environmental contamination have been reported and very few studies have investigated the presence of this parasite in water. In one study very low levels of *Cryptosporidium* oocysts in surface water in South Africa were found (Kfir et al., 1998); only a few oocysts were detected in drinking water (1 oocyst/10 ℓL) with a prevalence of 1.1%. In contrast to this, in a more recent study, a high number of *Cryptosporidium* oocysts were found in three rivers in South Africa; the Moos River in Mpumalanga, the Skeerpoort River in North West province and the Klip River in Gauteng province. *Cryptosporidium* oocysts were present in all three rivers with an overall prevalence of 43% (Duhain, 2011). However none of these rivers are in close proximity of our study area.

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The poor water supply system in Bushbuckridge municipality, consisting mainly of both surface and ground water, may contribute to the contamination of water with faeces from livestock and human and may therefore pose a potential risk for these communities. Few people (16%) have access to tap water (standpipe, yard or household connections) (Raab et al., 2008). The rest of the community relies on alternative means, such as nearby rivers, irrigation canals, dams and wells. These sources are also used by livestock for drinking and bathing, and occasionally dead animals have been found in the same water (Raab et al., 2008). The use of these alternative sources of water has led to water-borne illnesses, such as cholera, which occurred in 2008/2009 in Mpumalanga Province, including our study area. Initially the cases were directly linked to cholera outbreaks in neighbouring Zimbabwe, but a rapid increase within the province was due to local transmission and infections resulting from contamination of water supply systems and the spillage of raw sewage into rivers (Archer et al., 2009). This cholera outbreak suggests that unhygienic living conditions and improper use of wastewater may be commonly practiced in our study area and may therefore favour the transmission of other water related diseases such as cryptosporidiosis (Gideon et al., 2007; Muchiri et al., 2009) and microsporidiosis (see Appendix 1).

In addition the widespread use of use pit latrines in rural communities of our study area, with no access to any level of formal sanitation may be considered as an important potential route of transmission for cryptosporidiosis. These pit latrines are not routinely emptied; consequently, flies gather and breed in the latrines and during heavy rains overflow of sewage may occur (Raab et al., 2008).

During this research no environmental sampling and therefore no proper investigation of the importance of water, food and pit latrines in the transmission of cryptosporidiosis in our study area was carried out. It is therefore necessary to identify *Cryptosporidium* species in environmental samples and to assess its significance as a potential risk factor to people, livestock and wildlife within our study area in future studies.

In addition, contacts between wildlife-livestock and between livestock-humans as a potential risk factors for cryptosporidiosis was assessed during this study. The probability of contact between cattle and different wildlife species crossing the boundaries of the KNP, was estimated to be highest for buffalo and impala. Therefore, transmission of

Cryptosporidium oocysts or other pathogens from wildlife to cattle may potentially occur. This hypothesis is of interest since zoonotic cryptosporidiosis (*C. ubiquitum*) was detected in two impala and one buffalo along the boundaries of the KNP and the disease may therefore be considered a potential threat to the rural communities.

The probability of contact between livestock and humans was investigated as potential risk factors for *Cryptosporidium* infections in children, but no significant associations were found. *Cryptosporidium* infections in Nigeria (Akinbo et al., 2010), Egypt (Helmy et al., 2013), China (Wanga et al., 2011), India (Khan et al., 2010), and Australia (Ng et al., 2012) have previously been related to animal contact. However, in this study the relatively small sample size and overall low prevalence levels may have been responsible for the failure to identify potential risk factors.

Although our study showed a low prevalence of cryptosporidiosis in animals and humans living at the interface of the KNP, the poor sanitation and water services in Bushbuckridge municipality and consequently the poor hygienic living conditions (unsafe drinking water and spillage of sewage) indicate that the communities are vulnerable to water or food-borne illnesses (Archer et al., 2009). In addition the partially permeable fence which separates the KNP from communal areas, allows the movement of wildlife outside the park, as well as the crossing of cattle into the KNP, and therefore transmission of *Cryptosporidium* species between wildlife, livestock and humans may occur.

CONCLUSIONS

This study investigated the prevalence of *Cryptosporidium* infection in wildlife, cattle and humans living at the KNP interface and identified different species and genotypes circulating within each compartment. The study also assessed potential contacts between cattle and wildlife that could increase the risk of transmission between wild and domestic species. Finally, the molecular identification of *Cryptosporidium* species allowed assessment of the zoonotic importance and public health significance of cryptosporidiosis in human communities at the wildlife/livestock/human interface of KNP.

FINDINGS WITH RESPECT TO RESEARCH QUESTIONS

1. Is *Cryptosporidium* circulating in free ranging wildlife in the KNP and adjacent protected areas, and if so at what level?

Cryptosporidium oocysts were detected in buffalo, impala and elephant, and a higher prevalence was found adjacent to the boundaries of the KNP than in the centre of the park. Although overall prevalence was low, this suggested that wildlife, specially buffalo and impala, can be exposed to *Cryptosporidium* oocysts from livestock and humans living at the KNP interface. Subsequently molecular analysis detected *Cryptosporidium* species in faecal samples from buffalo and impala but identified none in faeces from elephants. Therefore we have evidence that few *Cryptosporidium* species (*C. ubiquitum* and *C. bovis*) are circulating in buffalo and impala in the KNP, but this has not been demonstrated in elephants.

2. What species and genotypes of *Cryptosporidium* occur in wildlife and livestock at the edge of the protected area, and is there evidence for transmission between the two compartments?

In buffalo and impala, one *Cryptosporidium* species of zoonotic importance (*C. ubiquitum*) and one bovine-specific species (*C. bovis*) were detected, while only bovine-specific species were identified in cattle. The finding of the same bovine-specific genotype in both wildlife and cattle, gave indirect evidence for transmission between these two compartments, supported by the fact that contact was reported between cattle and wildlife (buffalo and impala).

3. Is cryptosporidiosis an important zoonosis in human communities living close to the KNP?

Cryptosporidium species detected in humans were predominately of anthroponotic origin. This showed that animals are likely to play a minor role in the transmission of cryptosporidiosis to humans in this study. However one zoonotic species (*C. ubiquitum*)

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was detected in wildlife and another (*C. meleagridis*) in humans, therefore a potential zoonotic threat to these communities cannot be ruled out, especially considering that HIV prevalence in people of this area is high. Despite the risk of *Cryptosporidium* transmission between wildlife livestock and humans in our study area is likely to be low and seems to occur only occasionally, this situation might be different in other areas of the KNP interface or in other situations, with higher densities of animals or different water dynamics. Therefore, further studies are necessary to confirm if our observations are localised or are representative of a large part of the KNP interface.

LIMITATIONS OF THIS STUDY

The main limitation of this study was the low number of samples that were analysed with highly sensitive and specific diagnostic tests, due to budget constraints. The validity of MZN and IFA would have been better understood if all samples had been analysed by PCR and results compared. The larger number of results would have allowed estimation of the sensitivity and specificity of the tests and of the prevalences in the populations using a Bayesian framework. The small sample size also limited our ability to identify potential risk factors using multivariable methods.

A further limitation of this study was the lack of concurrent sampling of water from rivers, surface and ground water within our study area could not be carried out. The detection of *Cryptosporidium* species from water samples would have given added insights into transmission of cryptosporidiosis between wildlife, livestock and humans.

In addition, seasonal patterns of cryptosporidiosis could not be assessed during this study and therefore the differences in prevalences during the dry and rainy season could not be determined. This limited our understanding of the importance of seasonality during this study and how the circulation and availability of water might affect the presence of the disease in different compartments.

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RECOMMENDATIONS FOR FUTURE RESEARCH

The epidemiology of cryptosporidiosis at the wildlife/livestock/human interface is complex and requires to be studied at different levels and with different methods. Our study was not able to respond to all the research questions. However it allowed us to obtain preliminary data and some relevant information to take into account when planning future studies on this topic:

Additional research is necessary to fully understand the epidemiology of *Cryptosporidium* spp. at the wildlife/livestock/human interface. Our study area provides suitable conditions for the spread of waterborne and foodborne diseases, including poor water and sanitation services, contact with wildlife and livestock and high prevalence of HIV infection. Therefore, we recommend that future studies should aim at detection of *Cryptosporidium* in environmental samples, including genotyping to identify the different species and genotypes. Sampling of the Sabie and Sand Rivers outside and inside the KNP and of surface water at different locations within each community should be carried out during the dry and rainy seasons in sources of water commonly used by people and eventually wild and domestic animals.

Further testing of wildlife, cattle and humans at the KNP interface is needed. The sample size should be increased and sampling should take place during both dry and rainy seasons. Associations between potential risk factors and *Cryptosporidium* infection in cattle, humans and wildlife should be studied.

There is also a need to further evaluate diagnostic tests for the detection of *Cryptosporidium* in wildlife, using larger numbers of samples, from multiple populations, tested with multiple tests. Finally sufficient funding should be available to test a large number of samples with molecular techniques, so that prevalences of *Cryptosporidium* spp. with that technique can be calculated at a population level.

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APPENDIX 1

Enterocytozoon bieneusi at the wildlife/livestock interface of the Kruger National Park, South Africa

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Short communication

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ABSTRACT

This study investigates the presence of *Enterocytozoon bieneusi* in domestic and wild animals living in the wildlife/livestock interface area of the Kruger National Park (KNP) in South Africa. Fifty fecal samples from domestic calves in rural communities and 142 fecal samples from impala (*Aepyceros melampus*) and buffalo (*Syncerus caffer*) in the KNP were analysed for *Enterocytozoon bieneusi*, using a nested PCR targeting the internal transcribed spacer of the rRNA gene. All wildlife samples were negative for *E. bieneusi*, whereas nine (18%) calf samples were positive. Three cattle specific genotypes (group 2) were identified, belonging to the known genotypes BEB4 and I, and one novel genotype (BEB3-like). One human-pathogenic genotype (D) was detected in one calf. This is the first study on microsporidia performed in a wildlife/livestock interface area of sub-Saharan Africa. Our findings show that at least one genotype of zoonotic importance is circulating in native cattle in the study area and the rest of the identified microsporidia were host-specific genotypes. Larger studies in domestic animals, humans and wildlife are necessary to assess the public health significance of *E. bieneusi* in that interface area.

1. Introduction

Microsporidia are obligate intracellular parasites with worldwide distribution in all major groups of animals and humans (Santín and Fayer, 2011). Among the four main human-pathogenic microsporidian species, *Enterocytozoon bieneusi* is the one most frequently diagnosed in humans and domestic and wild animals, infecting the enterocytes of the small intestine and causing chronic diarrhea. In humans, *E. bieneusi* has been reported to cause symptomatic and asymptomatic infections in immuno competent persons (Santín and Fayer, 2011). In immuno competent persons, the disease is usually self-limiting. However, in immunocompromised individuals, such as HIV-positive patients, in which the diseases was first described (Desportes et al., 1985), it can cause chronic and life-threatening diarrhea (Lores et al., 2002).

Molecular diagnostic tests have been used to identify microsporidia at a species level and the internal transcribed spacer (ITS) region of the rRNA has been used for the identification of *E. bieneusi* genotypes. Currently, at least 93 genotypes of *E. bieneusi* have been described, including 34 in humans, 11 in both humans and a variety of domestic and wild animals, 12 in cattle, 11 in pigs, and some other host-adapted genotypes in specific groups of animals (Santín and Fayer, 2011). Some of the *E. bieneusi* genotypes have broad host specificity and can be transmitted from animals to humans (Sulaiman et al., 2003).

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The same authors revealed an extensive genetic diversity of *E. bieneusi* genotypes in wildlife and concluded that many were related to or identical to those infecting domestic animals and humans. In addition to these genotypes with zoonotic potential, host adapted genotypes have been described in fur-bearing animals in Maryland, USA (Sulaiman et al., 2003). Recently, a standardized nomenclature for *E. bieneusi* genotypes based on the ITS sequence was introduced (Thellier and Breton, 2008). Five main groups, numbered 1–5, were segregated from the most divergent sequence EntCan A, which was isolated from a dog.

Group 1 contains all the genotypes isolated from humans so far, except one highly divergent genotype (CAF4) isolated from HIV-positive and HIV-negative patients in Gabon and Cameroon respectively (Breton et al., 2007; Santin and Fayer, 2009), which forms, together with some genotypes in non-human primates a distinct cluster, named group 5 (Li et al., 2011a).

Group 1, is the largest of all five groups and genotypes within this group have been isolated from a wide diversity of hosts worldwide, including humans and domestic and wild animals. A widespread genotype within this group is D, also identified as PigEBTS9, Peru 9, CEbC, PtEbVI and WL8. This genotype has been found in a large range of domestic and wild mammals including among others, pigs a *macaques* (*Macaca mulatta*), beavers (*Castor canadensis*), muskrats (*Ondatra zibethicus*), raccoons (*Procyon lotor*) (Buckholt et al., 2002; Santín et al., 2005; Sulaiman et al., 2003), foxes (Sulaiman et al., 2003), dogs (Lobo et al., 2012), falcons (Müller et al., 2008), horses (Santín et al., 2009), mice (Sak et al., 2011), and baboons (Li et al., 2011b). In cattle, genotype D was detected in the eastern United States (Santín et al., 2005) and South Korea (Lee, 2007). Genotype D has been reported in humans in Thailand (Leelayoova et al., 2006), Peru (Sulaiman et al., 2003), Cameroon and Gabon (Breton et al., 2007), England (Sadler et al., 2002), Niger and Vietnan (Espern et al., 2007), Malawi and Netherlands (Ten Hove et al., 2009), Brazil (Feng et al., 2011), Nigeria (Ayinmode et al., 2011), Russia (Sokolova et al., 2011), Portugal (Lobo et al., 2012), and Tunisia (Chabchoub et al., 2009).

Each of the other three groups of genotypes contain sequences isolated mostly from one host species: Group 2 includes genotypes isolated from cattle, such as J, I, BEB 3, BEB 4; group 3 is made up of three sequences genotypes from muskrats in USA and one isolate from a cat in Portugal; and group 4 comprises genotypes only found in raccoons (Santin and Fayer, 2009).

To date very few studies have been conducted to characterize *E. bieneusi* in cattle in developing countries. A significantly higher prevalence of *E. bieneusi* was reported in post-weaned compared to pre-weaned dairy calves in the United States (Santín et al., 2004). In North America and South Korea *E. bieneusi* genotypes were described in dairy calves and cattle (Fayer et al., 2003; Lee, 2007; Santín et al., 2005, 2004; Sulaiman et al., 2004), where six cattle-specific genotypes were reported: BEB1 or J, BEB2 or I, BEB3, BEB4, N, and M (Dengjel et al., 2001; Rinder et al., 1997; Sulaiman et al., 2004). In these studies, most genotypes were identified as cattle-specific (group 2 genotypes)

and very few isolates were genetically identical or similar to human genotypes, such as genotype D. Therefore, these studies concluded that only some *E. bieneusi* isolates from cattle may be of public health importance.

To the best of our knowledge, microsporidiosis in cattle has never been studied in sub-Saharan Africa, nor could we find any data on the presence of and characterization of *E. bieneusi* in cattle or wildlife species in South Africa. However, microsporidiosis has been reported in dogs and humans in this country (Dini et al., 1998; Samie et al., 2007). In the most recent study, Samie et al. (2007) detected *E. bieneusi* in 12.9% (33/255) of hospital patients and in 4.5% (3/67) of diarrheic primary school children by using a PCR-RFLP assay. However, no genotyping was conducted on those samples.

The goal of this study was to investigate the occurrence of *E. bieneusi* in domestic and wild hosts at the wildlife/livestock interface from the Kruger National Park (KNP) in South Africa using molecular methods to determine the circulating genotypes and their zoonotic potential.

2. Materials and methods

2.1. Study area

This study was conducted at the wildlife/livestock interface of the Kruger National Park (KNP), where interactions between wildlife and cattle have been reported despite the presence of a fence to separate wildlife in the KNP from livestock in adjacent rural communities (Brahmbhatt et al., 2012; Jori et al., 2011). Cattle were sampled at ten diptanks in the communal grazing lands in the Bushbuckridge area, the most populated region adjacent to the KNP. The diptanks were chosen according to their proximity to the KNP fence and were all located within 5 km from the KNP boundary. The study area for wildlife comprised three sites within the KNP and an adjacent private game reserve, as previously described (Abu Samra et al., 2011).

2.2. Sampling

A total of 50 fecal samples from the local Nguni type cattle, ranging from 6 to 12 months of age, were collected from 10 diptanks in the Bushbuckridge area. In addition, a total of 142 fresh fecal samples 71 from impala (*Aepyceros melampus*) ($n=71$) and 71 from African buffalo (*Syncerus caffer*) ($n=71$) living in the KNP and an adjacent game reserve were analysed during this study. In each of the three sampling sites within the KNP, buffalo and impala herds were followed and approximately 15–20 fresh fecal samples from each wildlife species were obtained. Samples were considered fresh when the surface of the droppings was still moist. All cattle and wildlife samples were collected between May/June 2008 and February/March 2009 and were transported immediately to the laboratory in ice-cooled containers and stored in 2.5% potassium dichromate at $4\,^{\circ}$C until processed.

2.3. DNA extraction

DNA was extracted from fecal samples (n = 142) using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, Ohio). Prior to the extraction the samples were washed three times with deionized water by centrifugation, and the pellet was transferred into a 2 ml tube containing lysing matrix E from the kit. After 1 ml of sodium phosphate buffer and 122 μl of MT buffer were added, the tubes were vortexed in a FastPrep instrument (MP Biomedicals, Solon, Ohio). The samples were processed further in accordance to the procedures recommended by the manufacturer.

2.4. PCR amplification and sequence analysis

A nested PCR targeting the ITS region of the rRNA gene of was used to detect *E. bieneusi* (Sulaiman et al., 2003). The secondary PCR products were purified using Montage PCR filters (Millipore, Bedford, MA) and sequenced using an ABI BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3100 automated sequencer (Applied Biosystems). The nucleotide sequences obtained were aligned with reference *E. bieneusi* sequences using ClustalX software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). The standard genotype nomenclature (Santin and Fayer, 2009) was used to identify *E. bieneusi* genotypes. Unique nucleotide sequences generated in the study were submitted to GenBank under accession numbers JQ923448-JQ923456.

3. Results and discussion

Of the 50 bovine fecal samples analyzed, nine samples (18%), originating from seven different diptanks, were PCR-positive. All PCR positive samples were sequenced to determine the genotypes of *E. bieneusi* present. Most *E. bieneusi* specimens identified, belonged to the cattle-specific Group 2; three isolates were identical to the BEB4 genotype, one isolate was identical to genotype I, and four isolates belonged to a new genotype, named as BEB3-like. The latter had a sequence 99.2% (241/243) identical to Cattle BEB3 (GenBank number: JQ923448). However, the sequence of one isolate was identical to the human-pathogenic genotype D (1/9). All 144 wildlife samples were negative for *E. bieneusi* by PCR.

This study reports for the first time the presence of *E. bieneusi* in indigenous domestic cattle living at the wildlife/livestock interface in South Africa with an estimated prevalence of 18%. This is slightly higher than the 15% prevalence of *E. bieneusi* found in cattle in South Korea (Lee, 2007) and somewhat lower than the 23% reported in cattle in the eastern United States (Santín et al., 2005). The detection of *E. bieneusi* in South African cattle shows the possibility that cattle living in rural communities excrete spores into the environment and are therefore a potential source of infection to other animals and humans.

Despite the presence of *E. bieneusi* in cattle, the parasite was not detected in impala and buffalo living in close proximity to cattle (72% of sampled animals) nor in those individuals sampled in the center of the KNP. Despite contacts between cattle and those wildlife species have been described in our study area (Brahmbhatt et al., 2012; Jori et al., 2011), no exchange of *E. bieneusi* was detected. This is not entirely unexpected, as most of the cattle were infected with bovine-specific *E. bieneusi* genotypes. In addition, our sample size was small, and therefore despite its presence was not detected in our study the possibility that impala and buffalo being natural hosts of microsporidia cannot be ruled out.

Most *E. bieneusi* genotypes found in this study appear to be cattle-specific (89%), while only one genotype (genotype D) of zoonotic importance was identified. This finding is in accordance with previous observations in other distant geographic locations: in eastern United States, 98.5% of *E. bieneusi* genotypes were identified as cattle-specific (J, I, BEB3 and BEB4) and few (1.5%) genotypes were human pathogens (genotype D) (Santín et al., 2005). Sulaiman et al. (2004) also found a high proportion (96.5%) of cattle-specific *E. bieneusi* isolates (J, I, BEB3 and -BEB4) from seven states in North America and from Portugal. In South Korea, cattle-specific genotypes of *E. bieneusi* were isolated in 90% of the positive animals: CEbA, I, J, and CEbF (with 99% similarity to J) (Lee, 2007). However, cattle-specific genotypes have also been reported in humans in China (I and J) (Zhang et al., 2011) and in Czech Republic (BEB4) (Sak et al., 2011). Genotype D, identified in cattle in South Korea and the United States, was likewise seen in the current study. Sulaiman et al. (2004) investigated age related prevalence of *E. bieneusi* in calves in the United States and revealed a significant higher prevalence in post-weaned than in pre-weaned calves. The present study targeted mainly post-weaned calves, however, in order to assess the effect of age on prevalence, a wider age range of calves, including pre-weaned calves, should be included in future studies.

This study demonstrates the presence of a zoonotic genotype *E. bieneusi* in cattle from the study area. Considering the high prevalence of HIV/AIDS in this area, the zoonotic importance of *E. bieneusi* should be studied more in detail, assessing its prevalence in the human population living in the same study area. This would contribute to a better understanding of the public health significance of this disease.

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