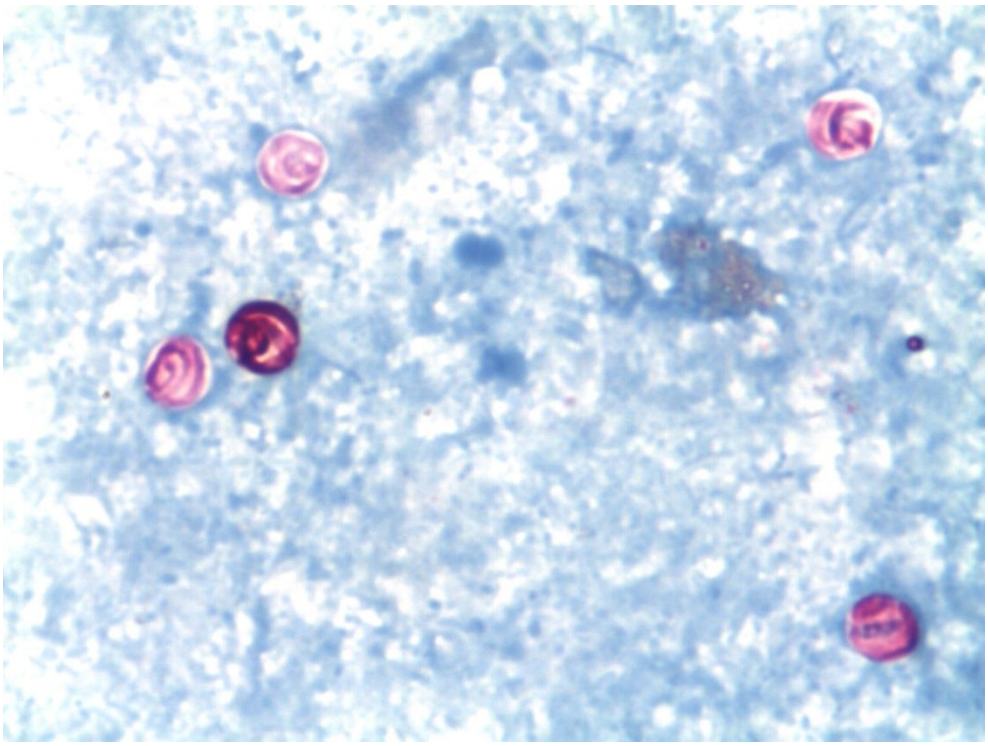
The prevalence and risk factors of *Cryptosporidium* infection among children in the Mnisi community, South Africa



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ABSTRACT

In the present study, the prevalence and risk factors of *Cryptosporidium* infection among children in the Mnisi community of South Africa were investigated. Stool samples of children under the age of five years old were collected from March to June 2012. For the diagnosis of *Cryptosporidium* spp. samples were analyzed with the modified Ziehl-Neelsen (ZN) technique. In addition, parents were asked to fill in a questionnaire to identify risk factors for *cryptosporidiosis. Cryptosporidium* spp. were detected in 5.6% (8/143) of the children and three samples were identified by polymerase chain reaction (PCR) as *C. hominis* (2/3) and *C. meleagridis* (1/3). No significant risk factors for the infection of *Cryptosporidium* were found, due to low prevalence, low significance of diagnostic tests used and the unequal distribution of samples for each risk factor. Further studies are needed to investigate risk factors of *Cryptosporidium* infection in children.

Keywords: *Cryptosporidium*, *cryptosporidiosis*, children, diarrhea, prevalence, risk factors, Mnisi community, sub-Saharan Africa, South Africa

INTRODUCTION

Apicomplexan protozoan parasites of the genus *Cryptosporidium* have a wide spectrum of hosts including domestic animals, birds, reptiles, fish, amphibians, wildlife and humans (Abu Samra *et al.* 2012a). Even though *Cryptosporidium* spp. has first been described in 1910, it was not until the 1970s when *Cryptosporidium* was recognized as an important cause of gastrointestinal disease in humans (Leitch and He 2011). Twenty six C*ryptosporidium* species and nearly 50 genotypes have been recognized and described and still new genotypes are being discovered (Abu Samra 2013). At least ten *Cryptosporidium* species and four genotypes can infect humans. *C. hominis* and *C. parvum* are globally the most commonly species infecting humans. Other species such as *C. meleagridis, C. cuniculis, C. suis, C. muris, C. canis, C. felis, C. ubiquitum and C. andersoni* have been occasionally found in humans (Abu Samra *et al.* 2012b).

Humans can acquire *Cryptosporidium* infections through several transmission routes such as person to person transmission (direct contact), zoonotic transmission (animals), foodborne transmission (ingestion of contaminated food) and waterborne transmission (water) (Xiao 2010). A single oocyst is sufficient to cause infection and disease. Oocysts are immediately infectious when excreted and are very stable and able to survive up to six months in a moist and cool environment. In water, oocysts remain viable in water for 140 days (Ramirez *et al.* 2004). In addition *Cryptosporidium* oocysts are very resistant to common water disinfectants, such as chlorine (Bouzid *et al.* 2013).

While *Cryptosporidium* infection in immunocompetent individuals usually occurs asymptomatic, *cryptosporidiosis* in children under the age of five and in immunosuppressed people, characteristically results in severe diarrhea. Nausea, vomiting, discomfort and low-grade fever are other clinical symptoms which may occur during an infection with *Cryptosporidium* (Bouzid *et al.* 2013). Symptoms in immunocompromised patients can be very severe and even death has been described (Adamu *et al.* 2014). In developing countries 45% of the children are experiencing an infection before the age of two (Mor and Tzipori 2008).

Because of the lack of treatment possibilities *Cryptosporidium* has the change to spread and distribute all over the world (Ryan *et al.* 2014, Desai *et al.* 2012). One drug, named Nitazoxanide, has been approved for children older than 12 years old. However, in immunodeficient people, no effect has been seen (Ryan *et al.* 2014, Abubakar *et al.* 2007).

The highest prevalence rate of HIV worldwide occurs in southern Africa and rural communities are especially affected. Therefore large number of immunocompromised people are susceptible to zoonotic diseases, such as *cryptosporidiosis* (WHO 2008).

Many studies in sub-Saharan Africa were performed with diarrheic children as their population and reported prevalence'-s varies considerably (Abu Samra 2013). 7.5% in Liberia, 12.5% in Guinea-Bissau, 14.8% in Congo (Mor and Tzipori 2008), 20% in Uganda (Akiyoshi *et al.* 2006), 18% in Zambia (Leav *et al.* 2002), 5.9% in Malawi (Morse *et al.* 2007) and 12.2% in South Africa (Abu Samra *et al.* 2012b). A prevalence of 8.6% was found in hospitalized diarrheic children in a recent study in Agincourt (Abu Samra *et al.* 2012b). The prevalence of *Cryptosporidium* is greatly compounded by HIV infection. Studies performed with HIV positive diarrheic children, prevalence'-s between 13.0% in Tanzania and 73.6% in Uganda were found. A prevalence of 24.8% and 12.5% among HIV positive children was found in Durban and in the Venda region of the Limpopo Province, South Africa (Leav *et al.* 2002, Adamu *et al.* 2014, Samie *et al.* 2006). Since the prevalence of HIV is high in developing countries, such as South Africa, studies on the epidemiology and zoonotic transmission of *Cryptosporidium* spp. is of great interest. Prevention of *Cryptosporidium* infection would be easier when zoonotic transmission routes are known. To identify zoonotic transmission routes, the Mpumalanga province in South Africa is an interesting study site, because of the close relation with the Kruger National Park (KNP).

Aim of the study

This study was conducted as part of a PhD project of Dr. N. Abu Samra. The overall aim of the PhD thesis was to evaluate the zoonotic importance of *Cryptosporidium* spp. at the wildlife/livestock/human interface along the boundary of the KNP in Mpumalanga Province, South Africa. This study involved sampling of cattle in twelve communities located within the wildlife, livestock and human interface and sampling of wildlife within the KNP.
The aim of the PhD project was to investigate the epidemiology of c*ryptosporidiosis* in wildlife, livestock and humans in a rural area in South Africa. Several research questions were addressed in the thesis and for this study, the following question was addressed: "Is *Cryptosporidium* an important zoonosis in human communities living close to the Kruger National Park?".
The aim of this study was to investigate the prevalence of *cryptosporidiosis* among children, younger than 5 years old, living at the periphery of the KNP.
Another aim was to investigate whether there is a relationship between several risk factors such as the age of a child, contact with cattle and other animals, water source, boiling water before drinking and the appearance of *Cryptosporidium* in feces. Hypothesis is that there is an association between a *Cryptosporidium* infection and the risk factors mentioned in the questionnaire.

Diagnosis

Symptoms of a *Cryptosporidium* infection are not pathognomonic, thus laboratory verification is required (Bouzid *et al.* 2013). Several techniques, for the identification of *Cryptosporidium*, have been developed, based on microscopic identification of *Cryptosporidium* oocysts (Ziehl-Neelsen (ZN) staining method), immunological methods (monoclonal antibody-based immunofluorescence assays (IFA) and Enzyme Linked Immunosorbent Assay (ELISA)) or molecular techniques (Polymerase Chain Reaction (PCR) (Omoruyi *et al.* 2014, Abu Samra 2013).
Omoruyi et al (2014) compared several diagnostic techniques for *Cryptosporidium* infection in HIV positive and HIV negative patients. Their study describes the sensitivity and specificity of the ZN staining technique, ELISA and PCR. Although the ZN technique is still considered as "the golden standard", a lower sensitivity was found, comparing to IFA, ELISA and PCR. However, the ZN technique has the advantage of being the only technique indicating active infections (Omoruyi *et al.* 2014, Abu Samra 2013). ELISA and PCR do not distinguish between active and non-active *Cryptosporidium* infections (Omoruyi *et al.* 2014). Furthermore, less cost is needed for using ZN technique (Abu Samra 2013). PCR has the advantage of improved sensitivity and specificity. 97-100% and 100% have been found for respectively the sensitivity and specificity of PCR technique (Omoruyi *et al.* 2014). However, due to the high costs, high technical expertise and infrastructure needs involved, these techniques have limited applicability. For the differentiation of *Cryptosporidium* spp. and thus the specific diagnosis of species/genotype, PCR is very useful (Abu Samra 2013).

Transmission of *Cryptosporidium*

Humans can acquire *Cryptosporidium* infections through several transmission routes such as person to person transmission (direct contact), zoonotic transmission (animals), foodborne transmission (ingestion of contaminated food) and waterborne transmission (water) (Xiao 2010). Putigani and Menichella (2010) geographically mapped the distribution of *Cryptosporidium* to provide an updated picture of the global parasite ecosystems. In developing countries more sporadic cases, rather than outbreaks, have been reported and in these countries *Cryptosporidium* infection have been related to the use of surface water as a main source of water and unhygienic and improper disposal of contaminated wastewater (Putigani and Menichella 2010). Several outbreaks caused by water contamination have been reported. In 1993, one of the largest human waterborne outbreaks occurred in Milwaukee, USA (Eisenberg *et al.* 2005).
For a long time modern diagnostic tools lacked the ability to differentiate between human-pathogenic and non-human-pathogenic species because the use of microscopy does not allow to identify *Cryptosporidium* at a species level. Therefore, the role of animals in the transmission of *Cryptosporidium* was still not entirely clear (Abu Samra *et al.* 2012a). Several outbreaks have been reported due to contact with infected calves (Gait *et al.* 2008, Kiang *et al.* 2006, Preiser *et al.* 2003), and *Cryptosporidium* has also been found in a wide range of wild animals which may serve as a reservoir for infection in humans (Oates *et al.* 2012, Abu Samra 2013).
Xiao (2010) describes molecular tools, such as PCR to detect and differentiate *Cryptosporidium* species. Small subunit rRNA (SSU rRNA) based tools are now generally used in genotyping *Cryptosporidium* spp. in humans and animals. The use of these new molecular tools led to a better representation of the zoonotic importance of *Cryptosporidium* spp. in animals and infection sources in humans (Xiao 2010, Ryan *et al.* 2014).
Since *C. parvum* was once considered to be the only species infecting humans, much attention has been given to zoonotic *C. parvum* (Mor and Tzipori 2008). Today, *C. parvum* is divided in *C. hominis* (once known as *C. parvum* genotype I) and *C. parvum* (once known as *C. parvum* genotype II) (Xiao 2010). *C. parvum* can be of anthroponotic origin (human to human transmission) and of zoonotic origin

(Xiao and Feng 2007). Unusual zoonotic species such as *C. meleagridis*, *C. canis* and *C. felis* have been described more frequently in humans from developing countries. However, in these countries, anthroponotic subtypes of *C. hominis* are the most common species reported to be responsible for human infections, while in industrialized countries *cryptosporidiosis* is mostly due to *C. parvum* (Xiao and Feng 2007).

MATERIAL AND METHODS

Study site and population

This study was performed in the Mnisi Traditional Authority (MTA), a rural area located in the north-eastern corner of the Bushbuckridge Municipal Area in the Mpumalanga province, adjacent to the Kruger National Park and therefore located within the wildlife, livestock and human interface. Human, livestock and wild animals live close to each other sharing playgrounds, grazing fields and water sources. The community consist of >40.000 people in which one animal clinic and eight health centers are located (Abu Samra 2013). It is mainly a Shangaan-speaking community, in which cattle farming forms an important agricultural activity. In 2010, a 35.1% prevalence of HIV infection was reported in the Mpumalanga province, a second highest prevalence of all provinces in South Africa (WHO 2008).

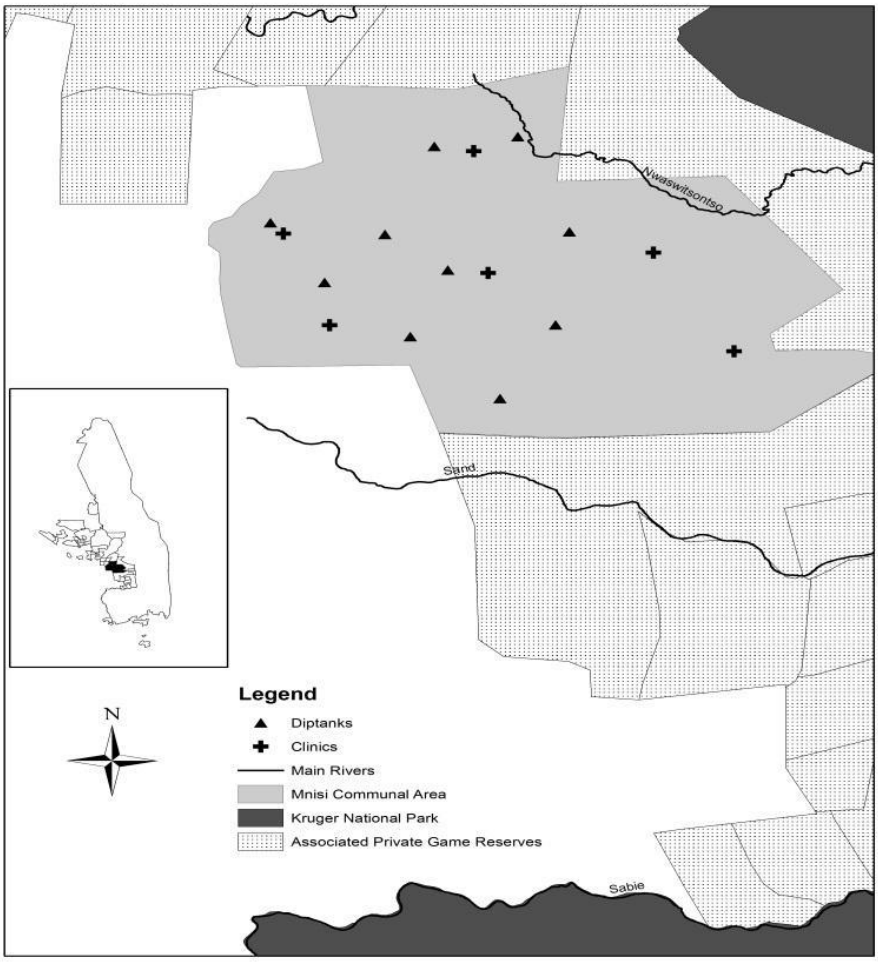

Figure 1: Study area for the location of clinics and diptanks where sampling took place (Abu Samra 2013).

Samples were collected from March to June 2012 at six participating clinics located in the rural area Mnisi (figure 1). Hluvukani clinic, Gottenberg clinic, Utha clinic, Islington clinic, Welverdiend clinic and Ludlow clinic are the clinics which participated. In a period of three months a total of 143 stool samples were collected from children under the age of 5 years old regardless of reported symptoms of diarrhea (table 1).

Name of the clinic	Amount of collected samples
Hluvukani	28
Utah	5
Islington	44
Welverdiend	23
Gottenberg	30
Ludlow	13

Table 1: The amount of collected samples from each individual clinic.

Sample collection, identification and preservation

Six clinics were visited to ask for their participation in this study. With a translator nurses were approached and informed about the importance of the study for their community. Information sheets were distributed through the clinic so visitors were aware of the study. Nurses were asked to approach every parent with a child under the age of 5 years old, regardless of reported symptoms, to collect stool samples of their child. Participation was voluntary and each parent who was willing to participate in this study was asked to sign a consent form. In addition parents were asked to fill in a questionnaire with relevant questions to identify risk factors for *cryptosporidiosis*. Each clinic was visited every second day to collect the stool samples. Samples were transported to the laboratory of Hans Hoheisen Wildlife Research Station (HHWRS), situated at the Orpen Gate of the Kruger National Park in the Mpumalanga Lowveld. Stool samples were diluted with potassium dichromate (2.5%) and then stored at 4 °C.
For the diagnosis of *Cryptosporidium* spp., samples were transported to the the Parasitology Reference Unit, National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. All samples were concentrated by the formalin ether sedimentation technique and analyzed with the modified Ziehl-Neelsen staining technique for detecting oocyst of all *Cryptosporidium* species. Using 50x and 100x objective microscopy respectively slides and oocytes were observed. See figure 2 for a microscopically positive control sample of *Cryptosporidium*. All samples positive with the modified ZN staining method were confirmed by PCR.

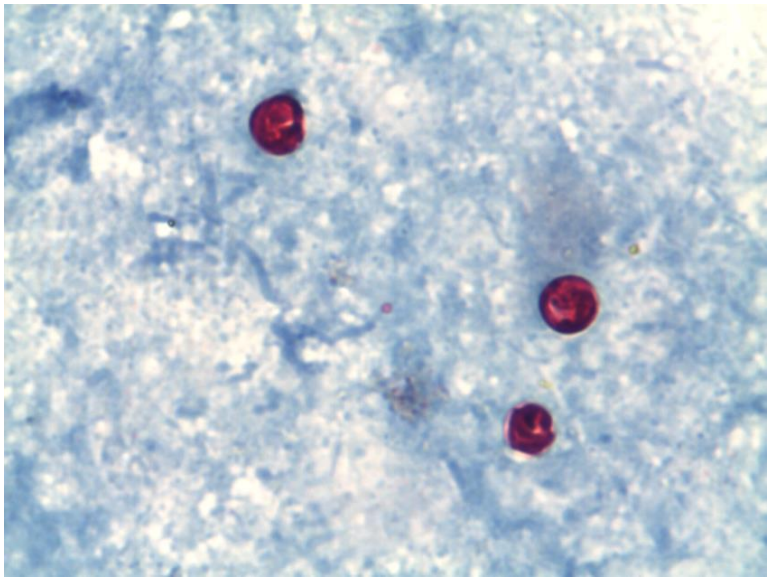

Figure 2: Positive control sample of Cryptosporidium.

Statistical analyses

Because of the small population Fisher's exact test was used for the univariable association between each potential risk factor and a *Cryptosporidium* infection. Statistical significance was set at $P < 0.05$. Because no significance was found, no further tests were necessary.

RESULTS

143 stool samples were collected from children under the age of five. From the 143 questionnaires, 141 questionnaires were completed and used to investigate the relationship between several risk factors and the appearance of *Cryptosporidium* infection. Question 7 from the questionnaire consisted of several questions, including the details of diarrheic episodes. This question is removed from the analysis of the results because it was mostly not or not completely filled in. Only the history of diarrhea since the child was born is included.

The age of the children from the collected samples were between 0 and 26 months old and is split into four groups (0-2 months, 2-6 months, 6-12 months and 12-26 months).

Out of the 143 samples, 8 were positive by the modified Ziehl-Neelsen technique and confirmed positive by PCR. Therefore the prevalence of *Cryptosporidium* infection in children is determined at 5.6% (95% CI= 2.4% - 10.7%). Table 2 shows an overview of the prevalence of *Cryptosporidium* found in this study. From each clinic, included in this study, at least one sample was found positive.

With 8%, the highest prevalence of positive samples for *Cryptosporidium* spp. was found within the age group of 6-12 months. The prevalence of positive samples for *Cryptosporidium* between the age of 0-2 months, 2-6 months and 12-26 months are respectively 6.7%, 3.8% and 4%. With a *p*-value of 0.765, no significance was found.

Contact with cattle has been reported in 33 children and within this group the prevalence of *Cryptosporidium* was 6.1%. Of the 108 children that did not come into contact with cattle, 5.5% was positive. With a *p*-value of 1.000, no significance was found.

136 children reported to drink water from the tap, while only seven reported to get their water from different sources, such as river or fountain. 5.9% of the samples were found positive within the group of children drinking water from the tap, while none of the children drinking water from different sources were found positive. With a *p*-value of 1.000, no significance was found.

Of the 24 children who drink boiled water, 4.2% was positive. 119 children did not drink boiled water and the prevalence is 5.9%. With a *p*-value of 1.000, no significance was found.

Of the 143 children, 84 had a history of diarrhea since they were born. The amount and severity of diarrhea is not taken into account. Within this group, 3.6% were found positive and 7.1% of the 54 children without a history of diarrhea were positive. With a *p*-value of 0.437, no significance was found.

The association between risk factors, investigated in this study, were not significant at a confidence level $\alpha = 0.05$.

Positive samples were also sequenced for the identification of *Cryptosporidium* species. 3 samples were identified as *C. hominis* and 1 as *C. meleagridis*.

Variable		Number of children participating	Amount of positive samples	Prevalence (%)	*p*-value
Age (in months)	0-2	15	1	6.7	0.765
	2-6	26	1	3.8	
	6-12	50	4	8.0	
	12-26	50	2	4.0	
Contact with cattle	Yes	33	2	6.1	1.000
	No	108	6	5.5	
Contact with other animals	Yes	105	6	5.7	1.000
	No	36	2	5.5	
Source of drinking water	Tap	136	8	5.9	1.000
	Other	7	0	0.0	
Boiling water before drinking	Yes	24	1	4.2	1.000
	No	119	7	5.9	
History of diarrhea	Yes	84	3	3.6	0.437
	No	56	4	7.1	

Table 2: Prevalence of Cryptosporidium spp. among 143 children in the Mnisi community and the univariable association of risk factors with Cryptosporidium infection.
*Reference level

DISCUSSION

Cryptosporidium spp. are worldwide recognized as important parasites causing severe diarrhea in children and immunocompromised individuals. Especially in young children, malnourished and immunosuppressed patients, *Cryptosporidium* infection can be life-threatening (Desai *et al.* 2012). Molecular techniques have been developed to diagnose *Cryptosporidium* at a species level, which improved our knowledge about the zoonotic transmission routes. Since the prevalence of HIV is high in South Africa, studies on the epidemiology and zoonotic transmission of *Cryptosporidium* spp. in this region is of great importance.

Many studies in children and immunocompromised people have been performed, suggesting that prevalence of *cryptosporidiosis* in developing countries is higher than in industrialized countries (Abu Samra 2013). Most studies in the sub-Saharan Africa involved diarrheic children. Various prevalence'-s have been reported: 7.5% in Liberia, 12.5% in Guinea-Bissau, 14.8% in Congo (Mor and Tzipori 2008), 20% in Uganda (Akiyoshi *et al.* 2006), 18% in Zambia (Leav *et al.* 2002), 12.2% in South Africa (Abu Samra *et al.* 2012b) and 5.9% in Malawi (Morse *et al.* 2007). In studies performed with HIV positive diarrheic children, prevalence's between 13.0% in Tanzania and 73.6% in Uganda were found. A prevalence of 24.8% and 12.5% among HIV positive children was determined in Durban and in the Venda region of the Limpopo Province, South Africa (Leav *et al.* 2002, Adamu *et al.* 2014, Samie *et al.* 2006). In the present study, a prevalence of 5.6% (95% CI= 2.4% - 10.7%) was found, which is in accordance with a recent study close to our study site, which reported a prevalence of 8.6% in hospitalized diarrheic children (Abu Samra *et* al. 2012b).
In this study all samples were analyzed by the modified ZN staining method, which has been reported to have a low sensitivity (46.2%) (Omoruyi *et al.* 2014). For this reason false negatives could have been emerged, which may have caused a lower prevalence found in the present study.

Species found in humans in developing countries are predominantly of anthroponotic origin, which is in accordance with our findings. However, *Cryptosporidium* species of zoonotic origin have been described previously in HIV patients (Xiao 2010). And therefore, in our study area, where HIV prevalence is high and livestock and human live closely together, a zoonotic transmission route may have been expected. However, the appearance of *C. meleagridis* in this study may suggest zoonotic transmission occurs.

No association between potential risk factors and *Cryptosporidium* infection has been found. This may be due to the fact that sample size was small. In ideal circumstances, when the amount of samples taken would have been equally divided over both groups (e.g. yes and no contact with cattle), significance (with a p-value <0.05) could only be found if all eight positive samples were found in one group, indicating the low statistical power of the study with so low a prevalence.
In a few groups, for example water source, numbers were very unequally divided between the two groups, which made statistical power even lower. Other limitations in the data collection may have been reduced the study power too. As mentioned above, due to a limited budget, highly sensitive diagnostic tests, such as PCR, were not available and the use of the modified ZN technique may be responsible for a number of false negative samples.
Also, during sampling, there was no supervision of what information was told to the parents by the nurses, how samples were taking and how samples were preserved. And it is unknown what percentage of distributed sample material were actually brought back and is included in this study.

Even though we did not find any significant risk factor, due to the low statistical power, we cannot conclude whether there is no zoonotic transmission and therefore further investigation is needed. However, findings and limitations of the present study can be used to improve further research. If significance can be determined in upcoming studies, it might be possible to reduce the prevalence of

Cryptosporidium infection by preventive measurements such as the limitation of human-animal contact, limiting the movement of livestock, prevent contamination of human living areas, separating water sources from animal facilities and increasing hygiene measurements in any setting.

In conclusion, this study did not find any significant risk factors for the infection of *Cryptosporidium* in children in Mnisi community, investigating the relationship between risk factors and the prevalence of *Cryptosporidium* spp., due to low prevalence, low significance of diagnostic tests used and the unequal distribution of samples for each risk factor.

The prevalence of HIV in our study area is one of the highest worldwide and the epidemiology of *Cryptosporidium* spp. at the interface of the KNP is complex. Therefore further studies on the zoonotic transmission of *Cryptosporidium* spp. are needed to better understand the route of transmission between animals and human.

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