PARASITIC INFECTION AMONG HIV/AIDS PATIENTS AT BELA-BELA CLINIC, LIMPOPO PROVINCE, SOUTH AFRICA WITH SPECIAL REFERENCE TO *CRYPTOSPORIDIUM*

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Abstract. Intestinal parasitic organisms are common pathogens among HIV patients worldwide and have been known to cause severe and life-threatening diarrhea in such subjects. In the present study, the prevalence of *Cryptosporidium* spp and other intestinal parasites in stool samples from 151 HIV/AIDS patients attending a HIV treatment center in South Africa was determined using standard parasitological methods, as well as molecular methods including PCR and quantitative PCR for confirmation of *Cryptosporidium* spp. In addition, the loop-mediated isothermal amplification (LAMP) method was evaluated for detection of *Cryptosporidium* spp in 24 stool samples. Standard parasitological methods indicated that *Cryptosporidium* spp (26.5%), *Entamoeba* spp (26.5%) and *Giardia lamblia* (13%) were the most common protozoan parasites, while *Ascaris lumbricoides* (8%), *Schistosoma mansoni* (6%) and *Trichuris trichiura* (4.6%) were the most commonly found helminths. PCR, quantitative PCR and LAMP methods identified *Cryptosporidium* spp in 28% (30/106), 35% (53/151) and 58% (14/24) of the stool samples, respectively. Multiple infections (34%) were commonly found in the study population. Females above 45 years had the highest *Cryptosporidium* prevalence (58%). Prevention measures must be implemented in order to curb the negative impact of *Cryptosporidium*-causing diarrhea among HIV/AIDS patients in this region as well as other parasitic infections identified in this study.

Keywords: *Cryptosporidium*, diagnostics, epidemiology, HIV/AIDS, microscopy, PCR, South Africa

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INTRODUCTION

Over the last two decades, *Cryptosporidium* spp has emerged as an important opportunistic pathogen among HIV/AIDS patients worldwide (MacKenzie *et al*, 1994; Insulander *et al*, 2005; Cantey *et al*, 2012). This enteric pathogen has defied water and

health authorities by its ability to withstand chlorine disinfection and filtration. It has been the cause of multiple diarrhea outbreaks in developed and developing countries. In developed nations, cryptosporidiosis represents the major public health concern of water utilities. It also induces diarrhea in immuno-competent persons, even though it might last for a short period. In developing countries, diarrhea caused by *Cryptosporidium parvum* early in childhood may be associated with subsequent impaired physical and cognitive development (Guerrant *et al*, 1999; Creek *et al,* 2012). Although HIV-positive individuals have been described as being more susceptible to cryptosporidiosis, the detection of *Cryptosporidium* spp in Bela-Bela, South Africa, a resting place for travellers, is not known and this calls for research that can fill the information gap in this area.

A number of studies have investigated the prevalence and epidemiology of cryptosporidiosis in patients with HIV infection in different parts of the world (Arikan *et al*, 1999; Missaye *et al*, 2013). The results of these studies have presented estimates that differ quite markedly from one another, ranging from 0 to 100% with a median of 32%. Studies conducted in different regions of Brazil have shown that *Cryptosporidium* spp is associated with symptomatic intestinal disease in HIV-infected individuals and in children suffering from acute diarrhea, including those attending day care (Goncalves *et al*, 2006). In different regions of Brazil, studies involving patients with compromised immune systems either HIV-positive or not and children, with or without diarrhea, have shown different species of *Cryptosporidium* in their stools, with *Cryptosporidium parvum*, *C. hominis* and *C. meleagridis* being the species identified in Sorocaba, while *C. hominis* and *C. parvum* was found in Sao Paulo and Taubate, respectively (Araujo *et al*, 2008). In Kenya, Malawi, Brazil, the United Kingdom and Vietnam, the majority of HIV-infected individuals are more infected by zoonotic genotypes than non HIV-infected individuals (Gatei *et al*, 2003). In Limpopo Province, South Africa, Samie *et al* (2006) showed the presence of *Cryptosporidium* spp from hospitalized patients and primary school children. Some of the patients are HIV-infected individuals and the prevalence of *Cryptosporidium* spp is 12.9% among these patients. In KwaZulu-Natal Province, Moodley *et al* (1991) showed that *Cryptosporidium* spp is the second most common enteric pathogen isolated from children admitted to hospital with gastroenteritis in rainy season, with infection rates varying from 1.2% to 20.9%, and 10% of the children infected with *Cryptosporidium* spp died. In Tanzania, Houpt *et al* (2005) described a prevalence of 17.3% among HIV patients. In India the prevalence rate was 9.2% among HIV-positive individuals with diarrhea, while in USA oocysts were detected in 4% of AIDS patients (Morgan *et al*, 1998). However, the prevalence of *Cryptosporidium* infection has not been investigated among HIV patients in Limpopo Province, South Africa, despite being host to a high HIV prevalence in the country (Adonis *et al*, 2013).

Infection with *Cryptosporidium* spp is routinely detected by microscopic examination of stools for characteristic oocysts, but the numbers of oocysts shed in stool may fluctuate. Therefore multiple specimens may have to be examined if infection is suspected should the initial microscopic examination prove negative (Clark, 1999). Other methods that can be used include the detection of *Cryptosporidium* oocysts using the electron microscopy (Brasseur *et al*, 1998), and commercial enzyme immu-

noassay kits for detecting fecal antigens of *Cryptosporidium* spp (Tumwine *et al*, 2005). Molecular biology techniques such as PCR, PCR-restriction fragment length polymorphism (RFLP), DNA sequencing and phylogenetic analysis, as well as loop mediated isothermal DNA amplification (LAMP), are used to confirm microscopic examination and also to determine genotypes of the *Cryptosporidium* spp involved in the infection (Verweij and Stensvold, 2014). Apart from PCR as the method for detecting *Cryptosporidium* spp in feces and environmental samples, other currently available methods remain difficult and costly to conduct on a routine basis, restricting their use by diagnostic laboratories. The LAMP assay has been successfully used for the detection of various pathogens, including viral and bacterial infections as well as protozoan infections, such as trypanosomosis and bovine, canine, and equine piroplasmosis (Ikadai *et al*, 2004; Alhassan *et al*, 2007; Iseki *et al*, 2007; Thekisoe *et al*, 2007; Bakheit *et al*, 2008).

In the present study, stool samples collected from patients attending a HIV treatment center in Limpopo Province, South Africa were tested for *Cryptosporidium* spp as well as other parasitic organisms using microscopic and molecular methods including PCR, quantitative (q)PCR and LAMP.

MATERIALS AND METHODS

Study area and sample collection

Bela-Bela clinic was selected as the study site for collection of samples. This clinic services a large population from different areas of Limpopo Province, South Africa (Fig 1). This clinic is situated in an area that is recognized as the resting place for travellers. A total of 151 stool samples were collected from HIV/AIDS patients attending the clinic. Samples were obtained from different populations so that comparisons could be made among age groups, gender and geographical origin. Age and gender were supplied by the clinic. Study volunteers were given leak-proof containers for the provision of about 10 g of stool specimens, which were collected in the morning and transported on ice and processed within 4-6 hours or stored at -20ºC to be processed within the next 3-5 days. A portion of all specimens was stored at -80ºC for further analysis if required.

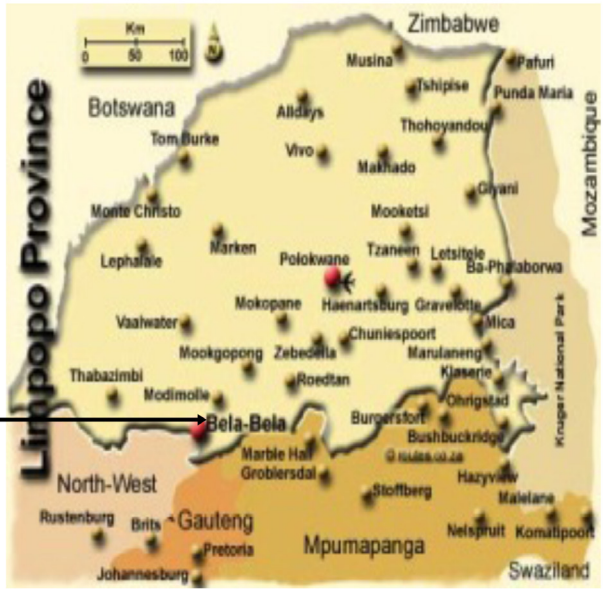

Fig 1–Map of Limpopo Province, South Africa, showing Bela-Bela, the study site.

The study protocol was approved by the Health Safety and Research Ethics Committee of the University of Venda, South Africa. Permission to use public health institutions was obtained from the Provincial Department of Health, Polokwane. Signed informed consent was obtained from all participants prior to collection of samples. Proxy consent was obtained from the parents or legal guardian

of minors. Specimens were tagged with research sample codes to protect the identity of the study volunteers.

Parasitological analysis by wet-mount technique

A thin smear prepared directly from the stool sample was treated with Lugol's iodine solution and observed under a light microscope (10X and 40X magnification) for presence of intestinal parasites, including *Entamoeba* spp and *Giardia* spp and eggs of *Ascaris, Diphyllobothrium, Hymenolepis, Schistosoma* and *Trichuris* spp.

Screening for *Cryptosporidium* oocysts with modified Ziehl-Neelsen technique

A thin smear of the stool specimen was air-dried and flamed for a few seconds for sterilization. Then the slide was exposed to formalin vapor and soaked in cold carbol-fuchsin solution. The slide was heated until steaming (but not to dryness) and was allowed to cool for 5 minutes. The slide was rinsed with tap water and decolorized with 5% sulfuric acid (about 1-2 minutes). The slide was rinsed with tap water, blot dried and examined under a light microscope (100X examination using oil immersion).

Detection of *Cryptosporidium* spp by PCR

Genomic DNA was isolated from *Cryptosporidium* oocysts as previously described with slight modifications (Haque *et al*, 1998). In brief, 50 µl aliquot of 1 M KOH and 18 µl aliquot of 1 M dithiothreitol were added to 250 mg or 250 µl of stool sample and the mixture was incubation at 65ºC for 15 minutes before being neutralized with 8 µl aliquot of 25% HCl and buffered with 80 µl aliquot of 2 M Tris HCl (pH 8.3). Genomic DNA then was extracted from the suspension using a QIAamp DNA stool Mini Kit (Valencia, CA). PCR amplification of *Cryptosporidium* 18S rDNA employed primers Cry 1 (5'-GCCAGTAGTCATATGCTTGTCTC-3') and Cry 2 (5'-ACTGTTAAATAGAAATGCCCCC-3') yielding an expected band size of 590 bp (Bushen *et al*, 2007). PCR was carried out in a total volume of 25 µl containing 0.6 µl of each primer (20 pmol/µl), 9.3 µl of distilled H_2O, 2.5 µl of 10X PCR buffer containing $MgCl_2$, 1 µl of dNTPs (10 mM each), 1 µl of *Taq* polymerase (Fisher Biotech, Bridgewater, NJ), and 10 µl of DNA template. Thermocycling (conducted in a T100™ Thermal Cycler; Biorad, Richmond, CA) conditions were as follows: 7.5 minutes at 95ºC; 50 cycles of 94ºC for 45 seconds, 53ºC for 75 seconds and 72ºC for 45 seconds; 72ºC for 7 minutes; and A final cooling step at 4ºC until analyzed. Each PCR assay included at least two positive controls (genomic DNA extracted from pure *Cryptosporidium* oocysts) and one negative control (distilled water). Both LAMP and PCR products were electrophoresed in a 1.5% Tris-acetic acid-EDTA (TAE) agarose gel and stained with ethidium bromide and visualized under UV light.

Detection of *Cryptosporidium* spp by qPCR

Cryptosporidium 18S rDNA was amplified using primers Crypt F (5'-CTGCGAATGGCTCATTATACCA-3') and Crypt R (5'-AGGCCAATACCCTACCGTCT-3') (Samie *et al*, 2006). The reaction was performed in a total volume of 25 µl containing 12.5 µl of 2X Maxima™ SYBR® Green Supermix (Fermentas, Glen Burnie, MD), 0.4 µl of each primer (20 pmol/µl), 6.7 µl of DNase-, RNase- and proteinase-free water (Fisher Biotech, Bridgewater, NJ) and 5 µl of genomic DNA. Thermocycling (conducted in an iCycler IQ, Biorad, Hercules, CA) conditions were as follows: 13.5 minutes at 95ºC, followed by 50 cycles of 15 seconds at 95ºC, 15 seconds at 60ºC and 20 seconds at 72ºC (with data collection at the end of each cycle). A thermal denaturation curve was generated by increasing

the temperature 0.5ºC step-wise 50 times. Each PCR experiment included at least two positive controls (genomic DNA extracted from pure *Cryptosporidium* oocysts) and one negative control (distilled water). Fluorescence was measured automatically by the iCycler during the annealing step of each cycle. The threshold cycle (Ct) was defined as the cycle number at which the threshold fluorescence was reached. Cycle numbers of each run were compared to a standard curve of known *C. parvum* DNA and transformed into oocyst numbers/g of stool sample.

LAMP assay for detection of *Cryptosporidium* spp

LAMP primer set was designed from SAM-1 gene of *C. parvum*, *C. hominis* and *C. meleagridis* using the primer explorer software (http://primerexplore.jp) as previously described (Bakheit *et al*, 2008). The LAMP reaction was performed in a final volume of 25 µl containing 12.5 µl of 2X reaction buffer (40 mM Tris HCl, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betaine, and 2.8 mM each dNTP), 1 µl (8 U) *Bst* DNA polymerase (New England Biolabs, Tokyo, Japan), 1.3 µl of primer mixture (40 pmol each of FIP and BIP primers, 20 pmol each of LF and LB primers, and 5 pmol each of F3 and B3 primers), 2 µl of DNA and 8.2 µl of distilled water. Positive DNA controls and negative control omitting DNA were included. Control and test DNA samples were incubated at 63ºC for 60 minutes and then heated at 80ºC for 3 minutes for termination of the reaction.

Statistical analysis

SPSS version 18 was used for statistical analysis. Chi-square test was used for comparison of the tests performed. A *p*-value of < 0.05 is considered statistically significant.

RESULTS

Population demographics and characteristics of stool specimens

A total of 151 samples were collected from 151 HIV/AIDS patients from Bela-Bela clinic, Limpopo Province, South Africa among whom 91 (60%) were females and 54 (36%) males. The gender was not known for the remaining 6 (4%) subjects. The study population was divided into 3 age groups: 1-24 (19 patients), 25-45 (96) and > 45 (34) years old. The ages of 2 participants were not known. Ninety-seven (64%) of the stool samples collected were diarrheal based on the physical bloody appearance (22, 15%), while the remaining were non-diarrheal.

Microscopic detection of *Cryptosporidium* species

Using the modified Ziehl-Neelsen technique, two different types of structures (oocysts) were observed, all of which were well stained with carbol fuchsin (red under light microscope). One type (40/151 samples) was of normal size (3-6 µm in diameter) while the other type (34 samples) was < 2 µm in size (Fig 2). Both structures were observed in 20 samples. In order to further differentiate between these two types of *Cryptosporidium* oocysts, PCR analysis was conducted, which revealed that only the bigger structures were *Cryptosporidium* while all samples that showed the smaller structures were negative both by conventional PCR and qPCR (see below). These smaller structures could either be fungi or microsporidia, which at times are stained by the method used.

Prevalence of intestinal parasites in the study population using standard parasitological methods

Using standard parasitological methods for identification of other intestinal

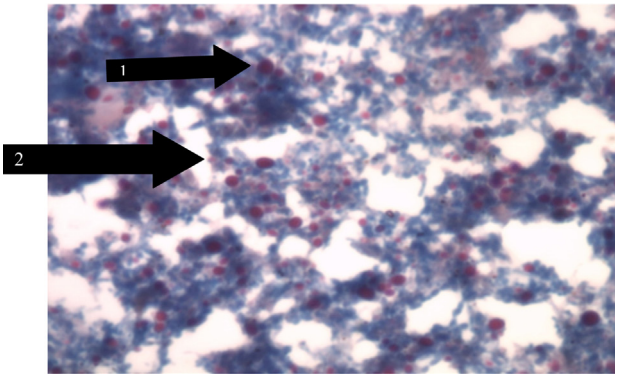

Fig 2–*Cryptosporidium* oocysts observed under light microscope. Stool smear was stained with carbol-fuchsin and viewed with 100X magnification. Arrow 1 shows a regular size *Cryptosporidium* oocyst and arrow 2 a stained "oocyst" of smaller size.

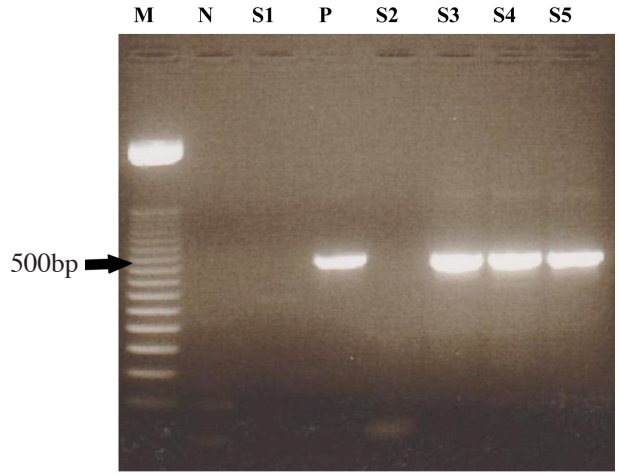

Fig 3–Agarose gel-electrophoresis of *Cryptosporidium* 18S rDNA 590 bp amplicons from representative stool samples of HIV/AIDS patients at Bela-Bela clinic, Limpopo Province, South Africa. PCR experiments are described in Materials and Methods. Lane M: 50 bp DNA size markers; lane P: positive control; lane N: negative control; lanes S1- S5: samples of which S1 and S2 are negative samples.

parasites in the 151 stool samples, the highest prevalence was *Entamoeba* spp (40, 26.5%), followed by *G. lamblia* (18, 11%). Only *Entamoeba* spp was detected in diarrheal stool samples (χ^2=4.166; p = 0.030) (Table 1). Although the prevalence of *G. lamblia* was also higher among the diarrheal samples, it is not statistically significant.

Forty-three (28.5%) samples had single intestinal parasitic infections, namely *Cryptosporidium* spp with the highest prevalence of 15 (10%), followed by *Entamoeba* spp of 9 (6%), *G. lamblia* of 6 (4%), *D. latum* of 5 (3.3%), *A. lumbricoides* of 3 (2%), hookworm and *S. mansoni* of 2 cases each (1%), and one case of *H. nana* (Table 2). In individuals < 24 years of age, single infection of hookworm were found, while there was only one case each of single infection of *Cryptosporidium*, *Entamoeba* and *G. lamblia* spp. Among individuals from 25 to > 45 years of age, *Cryptosporidium* was the most common single infection. There is no significant difference in the occurrence of single infections between diarrheal (29%) and non-diarrheal (28%) samples. However, *Entamoeba* spp single infections were mostly found in diarrheal (29%) compared with non diarheal (7%) samples.

Multiple infections were detected in 51 (34%) samples. The most common example of infection with two different parasites (total of 33 cases, 22%) was *Cryptosporidium* and *Entamoeba* (8 cases), while the most common among triple types of parasites (16 cases, 11%) was *Cryptosporidium* spp, *Entamoeba* spp and *G. lamblia* (2 female cases, 13%). Multiple infections were more common in females (33 cases, 36%) compared to males (16 cases, 30%). There was no association between multiple infections and age groups. Multiple infections with *Cryptosporidium* and *Entamoeba*

Table 1
Prevalence of intestinal parasitic infections in diarrheal and non-diarrheal stool samples collected from HIV/AIDS patients from Bela-Bela clinic, Limpopo Province, South Africa.

Parasitic organism	Diarrheal (n = 97) n (%)	Non-diarrheal (n = 54) n (%)	Total n (%)	χ^2; p-value
Cryptosporidium spp	24 (25)	16 (30)	40 (26.5)	0.426; 0.321
Entamoeba spp	31 (32)	9 (17)	40 (26.5)	4.166; 0.030
G. lamblia	13 (13)	5 (9)	18 (12)	0.567; 0.318
A. lumbricoides	5 (5)	7 (13)	12 (8)	2.891; 0.085
D. latum	10 (10)	8 (15)	18 (12)	0.671; 0.285
E. vermicularis	3 (3)	3 (6)	6 (6)	0.161; 0.486
T. trichiura	4 (4)	3 (6)	7 (5)	0.161; 0.486
S. mansoni	6 (6)	3 (6)	9 (6)	0.025; 0.591
S. haematobium	1 (1)	1 (2)	2 (1)	0.179; 0.589
B. hominis	1 (1)	2 (4)	3 (2)	1.273; 0.291
Hookworm	3 (3)	1 (2)	4 (3)	0.207; 0.549
Trichostrongylus spp	0 (0)	1 (2)	1 (1)	1.808; 0.358
H. nana	3 (3)	1 (2)	4 (3)	0.207; 0.549

Table 2
Distribution of single infections of intestinal parasites in stool samples of HIV/AIDS patients from Bela-Bela clinic, Limpopo Province, South Africa.

	Age groups (years)				Sample type		Sex		
Single infection	1-24	25-45	> 45	NA	D	ND	F	M	NA
Cryptosporidium spp	1	11	3	0	7	8	9	5	1
Entamoeba spp	0	6	3	0	7	2	6	3	0
G. lamblia	1	3	1	1	4	2	2	4	0
D. latum	0	3	2	0	3	2	3	1	1
A. lumbricoides	0	2	1	0	1	2	2	1	0
Hookworm	2	0	0	0	2	0	1	0	1
S. mansoni	0	2	0	0	2	0	2	0	0
H. nana	0	1	0		1	0	0	1	0
Total	4	28	10	1	27	16	25	15	3

D, diarrheal samples; ND, non diarrheal samples; F, female; M, male; NA, no data available.

spp (6 cases, 18%) were the most common among the diarrheal samples compared to the 2 (9%) cases in non-diarrheal samples. Among infections with three organisms, the two cases of infection with *Cryptosporidium*, *Entamoeba* sp and *G. lamblia* were found in diarrheal samples. Infections of *Cryptosporidium* with *Entamoeba* spp (3 cases, 14%); *D. latum* with *Entamoeba* spp (2 cases, 9%), as well as *Cryptosporidium* spp,

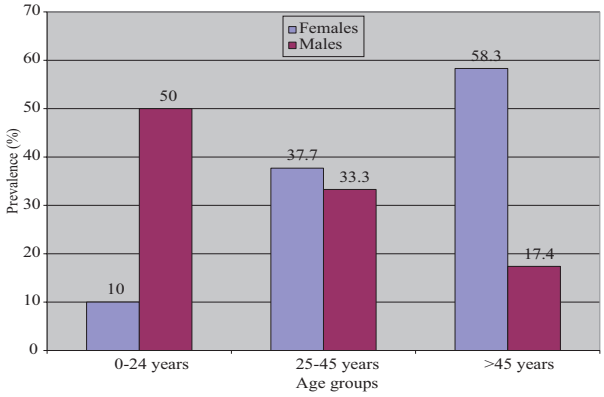

Fig 4–Prevalence of *Cryptosporidium* spp infection in HIV/AIDS patients at Bela-Bela clinic, Limpopo Province, South Africa according to age and sex. Presence of *Cryptosporidium* 18S rDNA in stool samples was determined by qPCR as described in Materials and Methods.

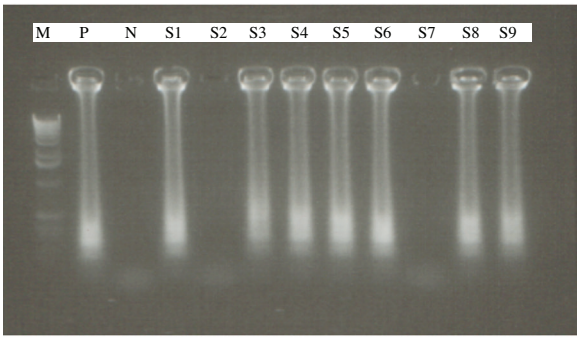

Fig 5–Agarose gel-electrophoresis of *Cryptosporidium*-specific amplicons from the LAMP assay. Experimental protocols are described in Materials and Methods. Lane M: 100 bp DNA size markers; lane P: positive control; lane N: negative control; lanes S1-9; samples.

S. mansoni with *A. lumbricoides*, *Entamoeba* spp, *G. lamblia* with *B. hominis*, and (quadruple infection) *A. lumbricoides*, *S. mansoni*, *E. vermicularis*, *Cryptosporidium* spp and *H. nana* (1 case each, total 14%) were identified in bloody diarrheal samples.

Detection of *Cryptosporidium* spp in stool samples analysed by conventional and qPCR

Due to insufficient reagents, only 106 samples were analysed for the presence of *Cryptosporidium* spp by conventional PCR and 30 (28%) were shown to be positive (Fig 3). On the other hand, qPCR analysis of all 151 samples revealed 53 (35%) positives. The distribution of *Cryptosporidium* spp among the different age groups or between males and females are not significantly different. It is of interest to note that while the prevalence of *Cryptosporidium* spp in females increased with age that in males decreased with age (Fig 4). Thirty-two (33%) of 97 diarrheal stool samples were positive for *Cryptosporidium* spp, with 7 (29%) positive among 24 diarrheal samples with blood. Of the 54 non-diarrheal stool samples, 21 (39%) were positive for *Cryptosporidium* spp. However, the prevalence of *Cryptosporidium* infection in diarrheal and non-diarrheal stool samples is not significantly different.

Detection of *Cryptosporidium* in stool samples by the LAMP method

From the 24 samples analysed by the LAMP method (due to the lack of reagents), 14 (58.3%) were positive for the presence of *Cryptosporidium* spp (Fig 5). Table 3 shows the results obtained by LAMP compared to those of real-time PCR.

Comparative analysis of microscopic, conventional PCR, qPCR and LAMP methods for the detection of *Cryptosporidium* spp in tool samples

A comparative analysis performed using 24 stool samples analysed by all 4 methods: microscopy, conventional PCR, qPCR and LAMP, for the detection of *Cryptosporidium* spp, 14 (58%) were positive for *Cryptosporidium* spp under light microscopy, with 6 cases of mixed infection of

Table 3
Comparison of four methods for detection of *Cryptosporidium* spp in 24 stool samples of HIV/AIDS patients from Bela-Bela clinic, Limpopo Province, South Africa.

Code	Sex	Age	Sample type	Blood in sample	Oocyst morphology	PCR	LAMP	qPCR	CT value
1G1	M	45	ND	no	big	1	1	1	23.47
1G3	M	41	ND	no	big	0	1	1	25.95
1G4	F	38	ND	no	big	0	1	1	23.49
1G5	M	52	D	no	small	0	0	0	-
1G6	F	39	ND	no	-	0	0	0	-
1G7	M	41	D	no	big	0	1	1	24.25
1G10	F	33	ND	no	-	0	0	0	-
1G11	F	35	D	yes	both	0	1	1	25.90
1G13	F	33	D	no	big	1	1	1	25.23
1G15	F	36	ND	no	big	1	1	1	26.52
1G17	F	46	D	no	both	0	1	1	25.99
2G8	M	53	D	no	-	0	0	0	-
2G15	M	30	D	no	-	0	0	0	-
2G25	M	NA	D	no	small	0	0	0	-
2G29	M	42	D	no	-	0	0	0	-
2G32	M	8	D	no	big	1	1	1	26.43
2G38	F	56	D	no	both	1	1	1	20.88
2G39	F	43	D	no	both	1	1	1	24.88
2G54	NA	39	ND	no	-	0	0	1	26.43
2G59	NA	7	ND	no	-	0	0	0	-
2G63	M	38	D	yes	big	1	1	1	21.64
2G64	M	51	D	yes	-	0	0	0	-
2G65	F	36	ND	no	both	0	1	1	25.80
2G67	M	1	D	no	both	1	1	1	25.49

1, positive; 0, negative, D, diarrheal; ND, non-diarrheal; NA, no data available.

large oocysts and small stained organisms suspected to be fungi or microsporidia; 8 (33%) by conventional PCR; 15 (62.5%) by qPCR; and 14 (58%) by LAMP (Table 3).

DISCUSSION

Cryptosporidium is one of the most common opportunistic infectious agents in HIV/AIDS patients causing severe and chronic diarrhea, representing an important cause of morbidity and mortality among HIV/AIDS patients (Araujo *et al*, 2008). The prevalence of *Cryptosporidium* spp varies from country to country as well as from region to region, but it has been identified to be more prevalent in developing countries. In most cases a high prevalence of *Cryptosporidium* spp has been reported among both HIV/AIDS and non HIV/AIDS individuals (Ojurongbe *et al*, 2011; Girma *et al*, 2014). Hitherto, the prevalence of this parasite in HIV/AIDS patients in Bela-Bela, Limpopo Province, South Africa is not known.

Diagnosis of this parasite requires well trained personnel to recognizing *Crypto-*

sporidium spp oocysts in stool samples. Microscopic examination of stool samples in this study identified *Cryptosporidium* (27%) and *Entamoeba* spp (27%) as the most frequent pathogens, either as single or multiple infections. However, in Jakarta, Indonesia *Cryptosporidium* (11.9%) and *C. cayetanensis* spp (7.8%) are the most frequent pathogens identified, either as single or mixed infections (Kurniawan *et al*, 2009). Mohandas *et al* (2002) found *Cryptosporidium* spp in 10.8% and *G. lamblia* in 8.3% of the samples in northern India. In Taiwan, Tsai *et al* (2006) reported a lower prevalence of *Entamoeba* spp (7.1%) in HIV-infected persons compared to that in this study (26.5%). In Madurai City, South India a higher prevalence of *Entamoeba* spp (37.5%) was reported by Ramakrishnan *et al* (2007) compared to our findings. In Cape Town, South Africa Adams *et al* (2005) showed high infection rates for *A. lumbricoides* (24.8%), *T. trichuiris* (50.6%) and *G. lamblia* (17.3%), but lower rates of *H. nana* (2.2%), *E. vermicularis* (0.6%), hookworm (0.08%) and *Trichostrongylus* (0.1%), as compared to our study. In Turkey, Buyukbaba *et al* (2004) showed high prevalence of *G. lamblia* (11.9%) among AIDS patients as compared to this study (9%). Reasons for these differences in intestinal parasite prevalence include environmental and behavioral patterns of peoples in the different regions.

Polyparasitism appears to be common among immuno-compromised (HIV/AIDS) individuals. Prevalence of multiple infections of 12.9% and 5% has been obtained by Kurniawan *et al* (2009) and Ramakrishnan *et al* (2007), respectively. Hailemariam *et al* (2004) indicated that 29.3% of patients had infections with two organisms, 4.8% with three organisms and 2.4% with four organisms. The current study found 10% of HIV/AIDS with infections of two or three organisms and 1% with four or five organisms. *A. lumbricoides* and *Blastocystis* spp are the most common multiple infections in the study of Hailemariam *et al* (2004), while the current study demonstrated the highest prevalence as being that of *Cryptosporidium* and *Entamoeba* spp. These findings indicate the diversity in the epidemiology of intestinal parasites among HIV/AIDS patients and further emphasize the need for epidemiological studies, which will help in the design of more effective control methodologies for these infections.

The LAMP method has been shown to have higher sensitivity compared to conventional PCR in the detection of *Cryptosporidium* spp in 270 stool samples from cattle, with zero and 33% positive by conventional PCR and LAMP, respectively (Bakheit *et al*, 2008). One of the reasons for the low sensitivity of conventional PCR is the presence of DNA polymerase inhibitors in the samples (Kourenti and Karanis, 2006), and elimination of such inhibitors in DNA samples would be critical if PCR assay method is to be applicable. In the present study albeit of only 24 samples, the LAMP method appeared to be as sensitive as qPCR and Ziehl-Neelsen staining. Further studies using larger sample size are needed to determine the applicability of the LAMP method in the detection of *Cryptosporidium* spp among HIV/AIDS patients as have been previously reported (Karanis *et al*, 2007; Bakheit *et al*, 2008). The major route of *Cryptosporidium* spp transmission is through water, particularly during the rainy season. This situation has been described in countries such as Mexico (Javier-Enriquez *et al*, 1997), Brazil (Newman *et al*, 1999), and Indonesia (Katsumata *et al*, 2000). In the present study, stool samples were collected during February until October, which covers both the rainy and the dry seasons in

South Africa. However, further studies must be conducted to identify the source of transmission of this parasite in the study region. The presence of enteric bacteria could indicate the presence of intestinal parasites. Water contamination with *Cryptosporidium* spp has been demonstrated in other parts of South Africa (Omoruyi *et al*, 2011). The occurrence of the *Cryptosporidium* spp through anthroponotic and zoonotic transmission cycles overlapping in the same area enhances the exposure of humans to this parasite, especially in areas with inadequate sanitary infrastructure. Limpopo Province is mostly rural and agrarian; it is possible that the transmission of this protozoan is through ingestion of water contaminated with animal feces. Therefore it is recommended that studies on zoonotic transmission be conducted in order to design preventive measures to reduce morbidity and other consequences of infections by these protozoan parasites in the Limpopo Province.

In conclusion, this study has demonstrated a high prevalence of *Cryptosporidium* infection among HIV/AIDS patients in Bela-Bela, Limpopo Province, South Africa. Implementation of affordable home-based water treatment methods, such as the use of ceramic filter, sand filtration, UV irradiation and ozone treatment could help reduce the threat of waterborne disease transmission, particularly cryptosporidiosis in HIV/AIDS patients in developing nations. Community education must be conducted regarding safe water provision and good hygiene practices in rural areas in order to prevent high prevalence of waterborne diseases. Research on the identification of the species of *Cryptosporidium* must be conducted. Furthermore, there is a need to conduct studies on the impact of multiple parasitic infections particularly in HIV/AIDS patients.

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