Cryptosporidiosis among HIV positive patients in the North Rift region of Kenya.

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Cryptosporidiosis among HIV positive patients in the North Rift region of Kenya.

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SUMMARY

Introduction: Cryptosporidiosis is a gastrointestinal illness characterized by watery diarrhea and caused by protozoan parasites belonging to *Cryptosporidium* species. It occurs in both humans and animals. In immuno-competent individuals the infection may resolve without intervention but in immuno-compromised individuals, it may persist to fatality.

Objectives: To determine the prevalence of cryptosporidiosis, cryptosporidium genotypes and associated factors among HIV patients.

Methods: This was a cross-sectional study, a total of 1794 stool samples were screened for C*ryptosporidium* oocysts, using modified ZN staining and parasite genotypes and sub-genotypes determined using PCR-RFLP analysis of the 18s RNA subunit and CP47 gene sequencing respectfully. Data was analyzed for measures of central tendency and statistical associations.

Results: The prevalence of cryptosporidiosis was 3.2% (58/1794) with no significant sex-related differences in risk of infection with *Cryptosporidium* (OR=1.2130, 95% CI=0.6895-1.341, P=0.5023). *C. hominis* was the most common genotype followed by *C. parvum* and then *C. meleagridis*. About 1% of the samples had a mixed infection of *C. hominis* and *C. parvum*. Cryptosporidiosis significantly associated with diarrhea (OR= 4.7087, P≤0.0001), headache (OR=2.218, P= 0.0033), and skin rash (OR=2.2179, P=0.0279).

Conclusion: The prevalence of cryptosporidiosis among HIV positive was of 3.2% and within the range of 3-4% reported in other parts of Kenya and within the East African region. Diarrhea, headache, low CD4 counts and skin rash were significantly associated with *Cryptosporidium* infection.

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Introduction

Cryptosporidiosis is a gastrointestinal illness characterized by watery diarrhea occurring in a wide variety of mammals, birds and reptiles [1, 2], and caused by *Cryptosporidium* species, apicomplexan protozoan parasites. While the infection can be resolved without intervention in immunocompetent individuals, cryptosporidiosis is increasingly becoming a major public health problem as an opportunistic infection in immunocompromised individuals, especially in HIV/AIDs [3]. Cryptosporidiosis is recognized as an emerging water- and food-borne disease that exerts considerable impact on pediatric health [4-6), and as an opportunistic infection in HIV- infected individuals [7-9]; and disease outbreaks have been associated with drinking and recreational water, farm animals [1] interpersonal interactions and environmental transmission [10]

In acquired immune deficiency syndrome (AIDS) individuals, the illness is characterized by chronic profuse watery diarrhea which may persist over a period of time, leading to weight loss, abdominal cramping, and death [11]. In such patients, the parasites may be present in the stomach, biliary and pancreatic ducts [12], and in the respiratory tract [13]. *Cryptosporidium* infections occur in both humans and animals, and therefore, zoonotic in nature. There are at least 16 *Cryptosporidium* species currently recognized [14], and of these, 6 (*C. parvum, C. muris,*

C. hominis, C. felis, C canis and *C. meleagridis*) are known to infect both humans and animals [1], and more importantly, immune-compromised individuals [15, 16, 8]. Thus, immuno-compromised individuals may be susceptible to infection with a wide range of *Cryptosporidium* species and subtypes. Therefore, a full understanding of the public health implications of *Cryptosporidium* infections, associated factors, and their significance in HIV infected individuals is necessary.

Materials and Methods

Study design: This was a prospective cross-sectional descriptive, health facility-based study carried out between January-December, 2006.

Study site and population: This study was conducted among HIV positive individuals who consented to take part in the study and seen at the Academic Model for Prevention and Treatment of HIV/AIDS (AMPATH) clinics at the Moi Teaching and Referral Hospital, Burnt Forest and Turbo Health Centres.

Collection of stool samples and demographic data: The subjects confirmed to be HIV positive were recruited into the survey. Fresh stool samples were collected in unused polypots from known HIV/AIDS individuals. The stool samples were screened for *Cryptosporidium* oocysts immediately or within eight hours after passage. *Cryptosporidium* positive samples were preserved in aliquots at -80^{o}C or in 2.5% potassium dichromate. Demographic data and individual's information on residence, housing, and sanitation was collected by use of a interviewer administered structured questionnaire.

Screening for oocysts: The stool samples were screened for *Cryptosporidium* oocysts using the modified Ziehl-Neelsen staining method [17].

Genomic DNA extraction from *Cryptosporidium* oocysts: Total genomic DNA was extracted from *Cryptosporidium* oocysts in stool samples that had been preserved in 2.5% potassium dichromate or kept frozen at -80^{o}C using the instructions in the manufacturer's manual supplied with the QIAamp® DNA Stool Mini Kit (Qiagen Ltd Crawley, West Sussex, United Kingdom). Briefly, a pea-size amount of formed stool or 200 µL of diarrheic stool was aliquoted into a 1.5 ml eppendroff tube, washed 4 times using 800 µL of distilled water prior to DNA extraction and centrifuged at 13000 rpm for 5 minutes. The pellet was then suspended in 1.4 ml of ASL extraction buffer supplied in a QIAamp kit. Oocysts were ruptured by subjecting them to a freeze-thaw cycle of -80°C for 30 min and -80°C for 15 minutes followed by thawing at 75 ^{0}C.

PCR amplification of *Cryptosporidium* genotypes: This was done using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 18s rRNA technique described by Morgan *et al*. [18]. The primary PCR used a forward primer 5'-TTC TAG AGC TAA TAC ATG CG-3' and reverse primer 5'-CCC TAA TCC TTC GAA ACA GGA-3'. The PCR reaction mixture contained 5 µl of Perkin-Elmer 10X PCR buffer (Norwalk, Conn, UK), 3 mM MgCl2, 240 mM (each) deoxynucleoside triphosphate (Promega), 250 nM (each) primer, 1.25 Units of *Taq* polymerase (Boehringer Mannheim, Livingston, United Kingdom), 4µg/µL of non-acetylated BSA (Sigma), and 0.5 µL of DNA template in a total 50 µL reaction mixture. A total of 35 cycles were carried out, each consisting of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute, with an initial hot start at 94°C for 3 minutes and a final extension at 72°C for 7 minutes. The PCR products were stored at 4 ^{0}C waiting to be used for the secondary PCR. The secondary PCR was performed in a total of 50 µL reaction mixture without BSA using 1 µL of the primary PCR product as template, forward primers 5´-GGA AGG GTT GTA TTT ATT AGA TAA AG-3´ and the reverse primer 5´-AAG GAG TAA GGA ACA ACC TCC A-3´ in a final concentration of 500 nM to amplify an 826 to 864 bp internal fragment. The concentrations of other reaction mix and amplification conditions for secondary PCR were the same as the primary reaction. Amplicons were analyzed by electrophoresis in a 2% agarose gel and visualized after ethidium bromide staining, and genotypes identified by RFLP analysis.

Restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA secondary PCR products was done by digesting 15 µL of the secondary PCR product in a total reaction volume of 40 µL consisting of 2µL (20 units) of *Ssp*1 (Promega) and 4 µL of 1X restriction buffer for species identification. The second set of restriction using *Vsp*1 (Promega), was

also performed in 40 µL volume except that 1 µL (10-12 units) of the enzyme was used. The restriction reactions were carried out in a water bath at 37°C for a minimum of 1 hour or overnight. The digestion products were separated on 2% agarose gel and visualized by ethidium bromide staining.

Subtyping of *Cryptosporidium* Genotypes: The CP47 Gene gene, a microsatellite gene located in chromosome 6 of *Cryptosporidium* species was used to subtype the *Cryptosporidium* species as described by Gatei *et al.* [19]. The following primers were used for a nested PCR amplification of a 380-500 bp fragment of the gene. For the primary PCR forward primer 5'-GCT TAG ATT CTG ATA TGGATC TAT-3' and a reverse primer 5'-AGC TTA CTG GTC CTG TAT CAG TT-3' were used, and for secondary PCR forward primer 5'-ACC CCA AGA AGG CGG ACC AAG GTT-3' and reverse primer 5'-GTA TCG TGG CGT TCT GAA TTA TCA A-3' were used. The primary PCR was performed in 50 µL volumes containing 1X PCR buffer, 3 mM $MgCl_2$, 240 µM of each dNTP, 250 nM of each primer, 4 µg/µL of non-acetylated BSA, 1.25 units of *Taq* polymerase and 0.5 µL of DNA template. Then 35 PCR cycles (94^{O}C for 45 seconds, 43^{O}C for 45 seconds and 72^{O}C for 60 seconds) were performed with an initial hot start at 95^{O}C for 3 minutes and a final extension step at 72^{O}C for 10 minutes. The secondary PCR was performed without BSA in 100 µL volumes using 2 µL of the primary PCR product and increasing the final concentration of primers to 500 nM while the rest of the ingredients remained at the same concentration as in the primary PCR. The PCR conditions remained the same except for the annealing temperature was adjusted to 55^{o}C. The PCR bands were stained with ethidium, visualized under the UV light and photographed. The PCR products were extracted and purified from the agarose gel using QIAquick® gel extraction kit and protocol as per the manufacturer's instructions.

The PCR products were sequenced in both directions using the CP47 specific forward and reverse primers on an ABI Prism 3730 automated Genetic Analyzer (Applied Biosystems). The nucleotide sequences were read using the Analysis Software (http://www.technelysium.com.au/ChromasPro.html) that uses the four colors to color-code analyzed data from all dye/virtual filter set combinations with adenine (A) as green, cytosine (C) as blue, guanine (G) as black, and thymine (T) as red in the electropherogram view.

Data storage and analysis: The data collected by use of the structured questionnaire microscopy and molecular biology techniques were stored and maintained in Microsoft excel 8.0. The data was analyzed by use of Epi Info 3.5.1 computer package for dispersion, central tendency, associations (P value of less than 0.05 were considered statistically significant) and multivariate analysis for statistically significant variables was done. The CP47 sequencing products were blasted to checked whether they corresponded to the CP47 sequences in the database using NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST) and subsequently aligned by the ClustalW program (www.ebi.ac.uk/Tools/clustalw2/) with manual adjustment [20]. Phylogenetic analyses were done on the aligned sequences to assess relationships among isolates and the distance-based neighbor-joining analysis.

Ethical issues: This research was approved by the Kenya Medical Research Institute's (KEMRI's) Scientific Steering Committee and the Ethical Review Committee, KEMRI, and the Moi University School of Medicine/Moi Teaching and Referral Hospital's Institutional Research and Ethics Committee (IREC).

Results

The Demographic Characteristics of Study Population: A total of 1794 HIV infected individuals whose demographic characteristics are shown in Figure 1.

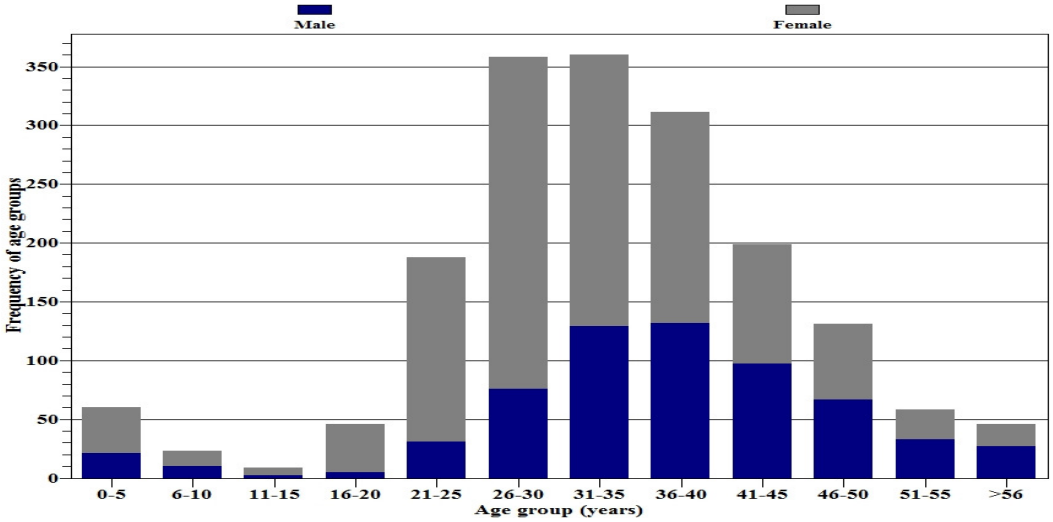

Figure 1: Frequency of age groups in years and by sex among HIV positive individuals.

The prevalence of cryptosporidiosis in the study population was 3.2% (58/1794). The prevalence of *Cryptosporidium* infection by sex was 2.9% (18/631) in males and 3.4% (40/1163) in females However, there was no significant difference between the sexes in terms of the odds of infection with *Cryptosporidium* (OR=1.2130, 95% CI= 0.6895 to 2.1341, P= 0.5023). About 3.3% (60/1794) of the study subjects comprised children aged 5 years and below with a cryptosporidiosis prevalence of 6.7% (4/60). The prevalence among those above 5 years of age was 3.1% (54/1734), a majority of which were between 21-50 years old. Prevalence of cryptosporidiosis revealed a bimodal seasonal pattern of distribution with a higher peak during the dry season (February–March) and a smaller peak (June - July).

The mean CD4 counts in the study population was 231.7 cells per microlitre. The mean CD4 counts among patients with cryptosporidiosis were 76.2 cells per microlitre, 3 times lower than in those without cryptosporidiosis. The logistic analysis showed that was a significant association of cryptosporidiosis and the CD4 counts (OR = 0.9918, 95% CI = 0.9874 - 0.9961, P = 0.0002).

Cryptosporidiosis and areas of residence: The prevalence of cryptosporidiosis was 7.3% (N=55) for the urban residents, 2.7% (N=635) for peri-urban residents and 3.5% (N= 1066) for rural residents. There was no significant association between location of residence and prevalence of cryptosporidiosis (OR= 0.9775, P= 0.9245). However, the odds of *Cryptosporidium* infection were 2.9 higher in the urban residents when compared to peri-urban residents (OR= 0.3507, 95% CI= 0.1138 - 1.0807, P= 0.0680) and 2.2 times in higher when compared to rural residents (OR= 0.4585,95% CI=0.1575 - 1.3347, P= 0.1526).

Cryptosporidiosis and water: About 16.6% of the study population drew their water from rivers or springs, 50.8% from wells or boreholes and 32.5% used tap water. The prevalence of cryptosporidiosis by sources of water was 3.4% (10/292) among river/spring water users, 2.8% (24/893) for well or borehole users and 4.2% (24/570) for piped water users. There was no significant difference in odds of *Cryptosporidium* infection between river/spring water users and well/borehole users (OR= 0.7788, P=0.5135), and tap water users (OR= 1.2396, P=0.5754), Table 1.

Table 1: Prevalence and statistical values for cryptosporidiosis according water sources and methods of water treatment in the study populations. (**UD = Undefined**)

		Cryptosporidium prevalence	Odds ratio	95% C.I	P-value
Water sources	Well/borehole	3.4%	0.7788	0.3680 - 1.6485	0.5135
	Tap water	2.8%	1.2396	0.5846 - 2.6282	0.5754
	River/spring	4.2%	reference		
Methods of water treatment	Sieving	0%	0.0000	UD	0.9730
	Chemical	2.3%	0.5424	0.1893 - 1.5545	0.2548
	No treatment	2.7%	0.6385	0.3676 - 1.1089	0.1112
	Boling	4.2%	reference		

About 43.4% (N=1754) of the study population boiled their drinking water, 0.8% sieved their water, 9.8% used chemical treatment, and 46.0% did not treat their water at all. The prevalence of cryptosporidiosis was 4.2% (32/762) amongst those who boiled their water, 2.3% (4/172) among those who used chemical (chlorination) water treatment for their drinking water, and 2.7% (22/ 807) in those who never treated their drinking water. The logistic regression output for the association of cryptosporidiosis and water treatment methods showed that the odds of infection were about 1.8 and 1.6 times higher in those boiling water compared to those using chemical treatment and those who did not treat their water respectively (Table 1).

Waste disposal: Approximately 90.2% (1579/1750) of the respondents used pit latrines, 9.2% (161/1750) used water closet toilet, and 0.6% (10/1750) used the bush for human waste disposal (Table 2). The odds of infection were 3.3 times higher in the group using the bush method for waste than those using the pit latrine (OR= 3.2628, 95% CI= 0.4058 – 26.2320, P= 0.2661) as shown in Table 2.

Table 2: Prevalence and statistical values of cryptosporidiosis in the study population in relation to methods of human waste disposal.

Method of water disposal	*Cryptosporidium* prevalence	Odds Ratio	95% C.I.	P-value
Pit latrine	3.3% (52/1579)	reference		
Flash toilet	3.1% (5/161)	0.9412	.03705 - 2.3910	0.8986
Bush	10.0% (1/10)	3.2628	0.4058 - 6.2320	0.2661

Cryptosporidiosis and diarrhea: The cryptosporidiosis prevalence among those with diarrhea was 6.5% whereas it was 1.5% in those without diarrhea. There was a strong association between diarrhea and the presence of cryptosporidiosis (P≤0.0001) (Table 3). The odds of having cryptosporidiosis were 4.7 times higher in those having diarrhea than in those without diarrhea. Among those aged five years and below, the prevalence of diarrhea was 43.3% with a prevalence of cryptosporidiosis of 11.5% while among individuals above 5 years of age 34.8% had diarrhea with a cryptosporidiosis prevalence of 6.3%. The prevalence among the non-diarrheic individuals is shown in Table 4. The odds of having cryptosporidiosis were 4.3 and 4.6 times higher in those with diarrhea among those aged 5 years and below and, those aged above 5 years respectively, Table 3.

Table 3: Prevalence of diarrhea and cryptosporidiosis in the study population.

Age	N	Prevalence			Statistical analysis		
		Diarrhea	*Cryptosporidium*		Odds ratio	95% CI	P-value
			Diarrheic	Non Diarrheic			
Whole group	1794	35.1%	6.5%	1.5%	4.7087	2.6524-8.3591	0.000
>5 years	1734	34.8%	6.3%	1.4%	4.6869	2.5911-8.4780	0.000
≤5 years	60	43.3%	11.5%	2.9%	4.3043	0.4211-44.0027	0.219

Among the diarrheic individuals, 323.2% (N=629) had diarrhea for less than one week with, 8.4% for between 1-2 weeks, 16.1% for more than two weeks, and 52.3% reported no diarrhea even though their stools were actually diarrheic on examination with cryptosporidiosis prevalence of 4.1%, 5.7%, 16.8% and 4.6% respectively as in Table 4. The logistic regression analysis for the association of *Cryptosporidium* infection with the duration of diarrhea showed that the odds of cryptosporidiosis were 1.4 times higher in patients with diarrhea for between one and two weeks and, 4.7 times in patients with diarrhea for more than two weeks (OR= 4.7222, 95% CI= 1.7915 - 12.4472, P= 0.0017) when those with cryptosporidiosis for up to one week are used as reference, Table 4.

Table 4: Prevalence of cryptosporidiosis among HIV positive patients with diarrhea and the logistic regression output for the association of cryptosporidiosis with the duration of diarrhea

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Duration of diarrhea	Number (%)	Prevalence	Odds Ratio	95% CI	P value
≤1 week	146 (23.3%)	4.1%	reference		
>1≤2 weeks	53 (8.4%)	5.7%	1.3725	0.3309 - 5.6926	0.6626
>2 weeks	101 (16.1%)	16.8%	4.7222	1.7915 - 2.4472	0.0017
Unknown Duration	328 (52.3%)	4.6%	1.1182	0.4250 - 2.9424	0.8209

Association between cryptosporidiosis and other health complaints

There was no significant association of fever (OR= 0.8993, 95% CI= 0.5152 - 1.5698, P= 0.7088), cough (OR= 1.0992, 95% CI= 0.6513 - 1.8548, P= 0.7232), vomiting (OR= 1.5479, 95% CI= 0.8375 - 2.8610, P= 0.1633) and abdominal pains (OR= 1.5784, 95%CI= 0.9298 – 2.6794, P= 0.0910) with cryptosporidiosis. However, the odds were about 1.5 times higher in patients presenting with vomiting and 1.6 times in those with abdominal pains compared to those without respective health complaints, Table 5. There was, however, a statistically significant association of skin rash (OR= 2.2179, 95% CI= 1.3044 - 3.7709, P= 0.0033) and headache (OR= 1.8087, 95% CI= 1.0664 - 3.0677, P= 0.0279) with cryptosporidiosis, Table 5. About 17.8% (320/1794) of the respondents did not have any health complaints presenting with a prevalence of 1.6% (5/320), while among those who had one complaint or the other the prevalence was it was 3.6% (53/1474). There was no significant association of cryptosporidiosis with health complaint (OR= 0.4256, 95% CI= 0.1688 - 1.0732, P= 0.0703) though the odds were about 2.3 times higher in patients with health complaints compared to those without any heath complaint.

Table 5: The statistical parameters and association of health complaints with cryptosporidiosis

Health complaints		Prevalence		Odds ratio	95% CI	P-value
Symptom	Status	Symptom	*Cryptosporidium*			
Fever	Yes	35.1%	3.0%	0.8993	0.5152- 1.5698	0.7088
	No	64.9%	3.3%			
Cough	Yes	47.7%	3.4%	1.0992	0.6513- 1.8548	0.7232
	No	52.3%	3.1%			
Skin rash	Yes	26.0%	5.4%	2.2179	1.3044- 3.7709	0.0033
	No	74%	2.5%			
Abdominal pain	Yes	32.8%	4.3%	1.5784	0.9298- 2.6794	0.0910
	No	67.2%	2.7%			
Vomiting	Yes	17.3%	4.5%	1.5479	0.8375- 2.8610	0.1633
	No	82.7%	3.0%			
Headache	Yes	42.7%	4.3%	1.8087	1.0664- 3.0677	0.0279
	No	57.3	2.4%			

Multivariate analysis

This was done among variables that were found to be associated with cryptosporidiosis namely CD4 count, diarrhea, skin rash and headache. This was done to elucidate how the variables were related to one another, and how they worked in combination in relation the occurrence of cryptosporidiosis. The multivariate outcomes in Table 6 show that diarrhea, headache and CD4 counts are predictors of cryptosporidiosis while skin rash is not.

Table 6: Predictors of cryptosporidiosis among HIV infected individuals

Predictors	**AOR**	**95% C.I.**	**P-Value**
CD4 count	0.9922	0.9922- 0.9922	0.0004
Diarrhea	3.1624	3.1624- 3.1624	0.0072
Headache	2.9893	2.9893 - 2.9893	0.0083
Skin rash)	0.9642	0.9642 - 0.9642	0.9346

AOR= adjusted odds ratio

The Circulating *Cryptosporidium* Genotypes

Out of the 58 isolates obtained 44 amplified, while 14 failed to amplify. The secondary PCR band products (826-864 bp) that are diagnostic of the *Cryptosporidium* species are shown in Figure 2.

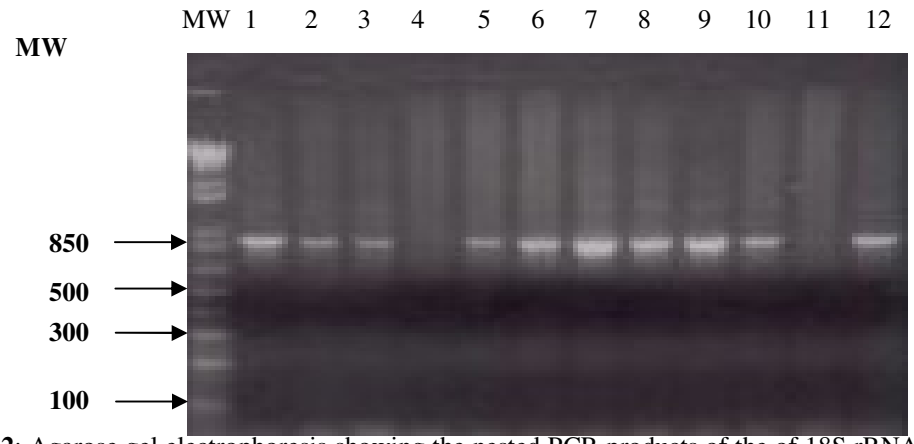

Figure 2: Agarose gel electrophoresis showing the nested PCR products of the of 18S rRNA gene (826 - 864 bp), diagnostic of *Cryptosporidium* species separated on 1.5% agarose gel stained with ethidium bromide. Lane 1: Sample A243, lane 2: A242, lane 3:A566, lane 5: C504, lane6: B216, lane 7: A659, lane 8: A594, lane 9: C537 and lane 10: A347and lane12: A3289. Samples in lanes 4 and 11 failed to amplify.

The RFLP analysis using *Ssp*1 enzyme, and *Vsp*1 restriction enzymes was used to identify the genotypes into *C. hominis*, which constituted 68.2% (30/44), *C. parvum* 18.2% (8/44) and *C. meleagridis* 13.6%6/44) of all the isolates. Of the *C. hominis* isolates, one was from the urban residence, 33.3% (10/30) were from peri-urban residences and 63.3% (19/30) were from rural residences while *C. parvum isolate*
(were seven from the rural residences and one from a peri-urban residence. *C. meleagridis* isolates were; two from peri-urban, 3 from the rural and one from urban residences.

Sub-typing of the *Cryptosporidium* Species

The nested PCR of the CP47 locus gave bands of 300-500 bp as shown in Figure 3

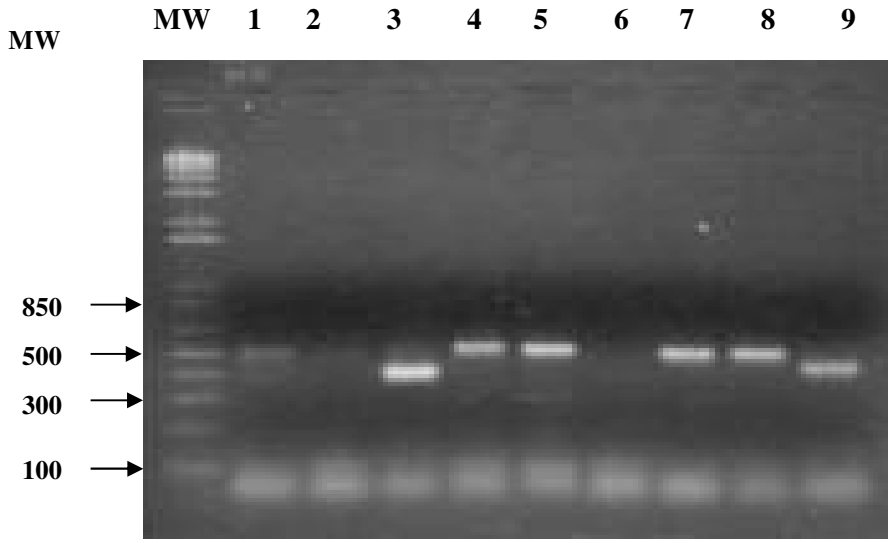

Figure 3: Agarose gel electrophoresis picture showing the 300-500 bp band products of the nested PCR of CP47 gene on 1.5% agarose gel stained with ethidium bromide. Lane 1:A659, lane 3: C292, lane4: C295, lane 5 C504, lane 6:B216, lane 7:A566 and Lane 9: A347 Samples in lane 2 and 6 failed to amplify.

Sequencing of the CP47 PCR Products
Of the 46 samples that were sequenced, 6 failed to amplify during sequencing while 5 amplified very short fragments that could not be blasted and another five were less than 300 base pairs and therefore could not be analyzed.

Sequence Alignment
The 30 samples that yielded clean CP47 sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

The aligned sequences (Appendix 1) were used to draw the phylogenetic trees.

Phylogenetic Tree
Based on aligned sequences of the CP47 gene the phylogenetic tree in Figure 5 was obtained which separated the thirty isolates into two distinct groups, *C. parvum*, eleven samples and *C. hominis*, nineteen samples.

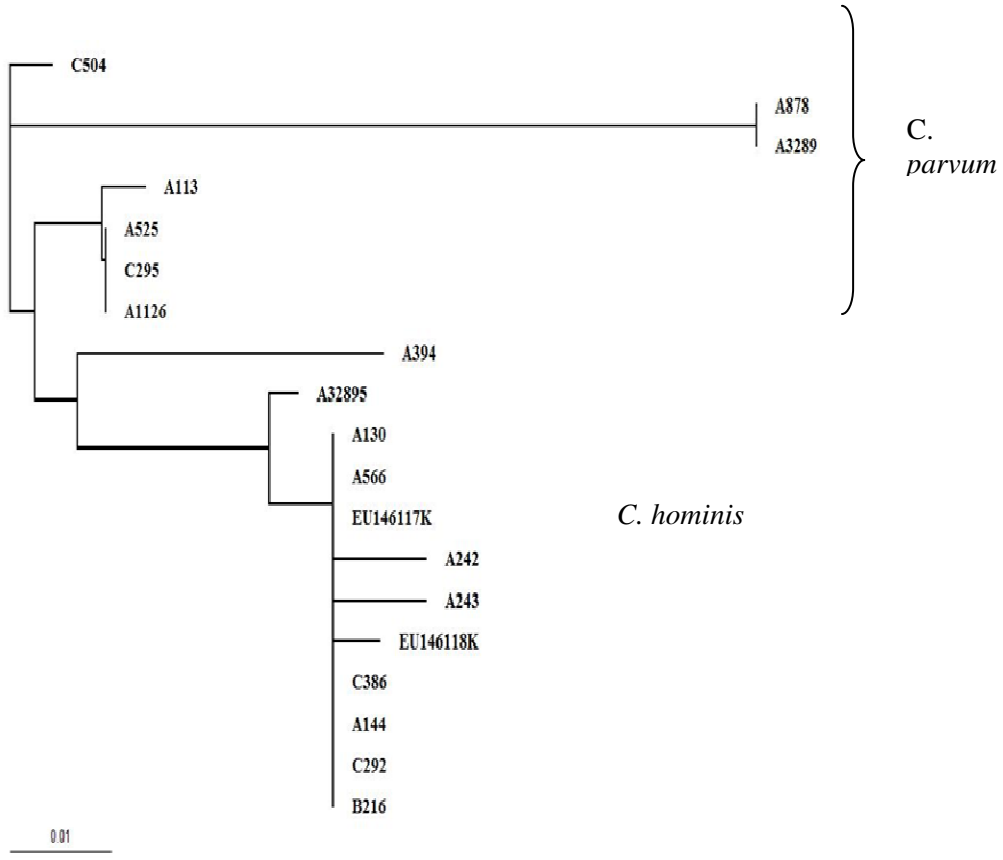

Figure 4: shows a clear distinction between the genotypes of *C. hominis* and *C. parvum* isolates from the study population. Samples EU146118K and EU146117k were *C. hominis* samples from Nairobi Kenya deposited in a gene bank by Gatei *et al*.2006

Based on sequence length polymorphism the *C. hominis* genotype was further divided into 6 sub-types A394, A32895, C292, B216, A242, A243, A144, A130, C386 and A566 while among the *C. parvum* had four sub-genotypes C504, A878 and A3289, C295, A113, A1126 and , A525.

Trinucleotide Analysis

This was done by assigning the CP47 locus of *C. hominis* as type I and *C. parvum* as type II and for the subtype identification, the trinucleotide TAA was coded as A and the TGA/TAG was coded as G with the following digit showing the number of each trinucleotide repeat. Ten subtypes within the *C. hominis* species were identified as IA43G18, IA48G23, IA52G28, IA52G29, IA53G29, IA53G31, IA54G31, IA55G32, IA56G33, and A57G32 while in the *C. parvum*, seven subtypes were identified as IIA30G12, IIA31G14, IIA32G14, IIA35G15, IIA36G16, IIA38G15and IIA37G18.

Discussion

Prevalence of cryptosporidiosis among the HIV infected persons was 3.2%, which falls within the range of 3-4% observed in Malaysia [9] and in Los Angeles, USA [21, 22] observed that cryptosporidiosis is highest in poorly developed countries because of poor hygienic and low socioeconomic standards. Prevalence of cryptosporidiosis was higher among the HIV infected children aged 5 years or less than in those HIV infection persons aged above 5 years old. The HIV infected patients who were five years or less had a higher risk of infection with *Cryptosporidium* than those HIV infected aged 5 years old and above. Though the study demonstrated a higher prevalence of cryptosporidiosis in children with HIV/AIDS, the finding was not statistically significant probably due the small number (60/1794) of HIV positive children that were captured during the study. Even then, these findings are in tandem with other reports in Thailand [21], Oregon [23], and in Los Angeles County [20] both in the USA which showed that cryptosporidiosis prevalence was higher in HIV infected children than in the HIV positive adults. The higher prevalence and risk of infection with *Cryptosporidium* in children is probably because children have greater susceptibility to infections attributable to an immature immune system, and the possibility of a fecal-oral transmission of the infections. There is need, therefore, for the health care providers and those involved in the management of HIV/AIDS in children to come up with novel ways of preventing and controlling diarrhea illnesses in HIV positive children, and the inclusion of cryptosporidiosis diagnosis as routine procedure in the examination of stool of diarrheic children.

Cryptosporidiosis prevalence of 3-44% among HIV/AIDS patients have been reported in Nairobi Kenya [24], Brazil [8], Venezuela [25], Iran [26], Nepal [27] and India [8] using the same diagnostic technique used in the current study. These trends of the reported burden of cryptosporidiosis suggest that each country or region could be unique due to the varied ecological conditions, human activities and socioeconomic factors that may influence the various routes of transmission

Prevalence of cryptosporidiosis revealed a bimodal seasonal pattern of distribution with a higher peak during the dry season (February–March) and a smaller peak (June - July). The transmission during both seasons was more of anthroponotic than zoonotic. This pattern was similar to that observed in Central Kenya [28], Brazil [29], and United States of America [30]. Elsewhere in the world, however, peak transmission is during hot and humid seasons [31]. Peak transmission seasons during the late summer have been observed in Canada [32], South Africa [33], and USA [34], whereas in Spain peak transmission of cryptosporidiosis occurred during autumn-winter period [35]. Results of the present study and previous studies suggest that seasonal prevalence of cryptosporidiosis varies from place to place probably due to differences in risk factors and determinants that may be unique for different regions, which in turn may influence the pattern of *Cryptosporidium* transmission.

The mean CD4 count among the cryptosporidiosis infected HIV patients was 76.21 cells per microlitre of blood as compared with the mean of 244.1 cells per microlitre among the cryptosporidiosis negative patients indicating that patients are more likely to contract *Cryptosporidium* infection with the lowering of the immune status as evidenced by the association of low CD4 counts and cryptosporidiosis. The mean CD4 counts were significantly different between diarrheic and non-diarrheic patients (P = 0.0002).

These findings concur with those in Iran [26] and in Nepal [27].

Diarrhea was significantly associated with the presence of cryptosporidiosis among the HIV positive patients (OR= 4.7, P≤ 0.002), There was a strong association of cryptosporidiosis and diarrhea and that the odds of infection were significantly higher in those patients who had diarrhea for more than two weeks compared to those who had it for less than two weeks, (P= 0.0017) and that it was more frequent in those living in the urban residences. The rural and urban residences were similar in the association of diarrhea with cryptosporidiosis. However, diarrhea was more frequent among the HIV positive children with a cryptosporidiosis prevalence of 11.7% and a significantly higher statistical chance of having cryptosporidiosis (OR= 4.3) compared to the HIV children without diarrhea. These results are in tandem with those reported in Argentina [36], Thailand [22] among diarrheic HIV positive children. The prevalence of cryptosporidiosis among diarrheic children in the present study is below that of 33.3% in Italy [37], Uganda [4] among HIV positive children with persistent diarrhea. The report from Uganda [4] is one of the highest rates reported, a trend that may be explained by the fact that the study used commercial kits based on direct-fluorescent monoclonal antibody test for the detection of *Cryptosporidium* oocysts that is more sensitive than the ZN staining microscopy that was used in this study. These studies therefore, give credence to the fact diarrhea illness is worse in HIV infected children because of the lowered immunity and the possibility of the presence of many other diarrheic agents that compound the episodes, hence the need of concerted efforts of preventing and controlling diarrhea in these children. There is furthermore there is need for a more comprehensive assessment of all possible opportunistic diarrheic agents in HIV patients to establish the complete picture of the etiology of diarrhea in HIV patients and the impact of the interaction of these agents on the severity of diarrhea and the progression of the HIV/AIDS syndrome.

Except for headache (OR=, 2.218, P= 0.0033) and skin rash (OR= 2.2179, P=0.0279), other health complaints such as fever, cough and abdominal pains did not show any statistically significant association with cryptosporidiosis. Headache and skin rash are common complaints among HIV patients and are therefore an indication of a declining immune status, which in turn is associated with increased chances of acquiring opportunistic infections including cryptosporidiosis as evidenced by the low CD4 counts among those patients infected with *Cryptosporidium* species in the current study. However, on multivariate analysis skin rash was found not to be significantly associated with cryptosporidiosis when considered with the other factors of diarrhea, headache and CD4 counts. These observations therefore indicate that it is difficult to diagnose cryptosporidiosis on basis of clinical symptoms alone.

The Circulating *Cryptosporidium* Genotypes and Sub-types

About 68.2% of the isolates were *C. hominis*, 18.2% *C. parvum* and 13.6% were *C. meleagridis*. These findings compare very well with those from Kenya, Malawi, Brazil, Vietnam, and United Kingdom [5], Dijon Hospital in France [38], Iran [39] and Haiti [40]. This suggests that person-to-person and probably environmental transmission is more common than zoonotic transmissions among these individuals. In contrast, the prevalence of *C. parvum* in parts of some European countries namely France, England and Northern Ireland is higher than *C. hominis* [41, 42, 43]. There are no explanations proffered so far, as to why the trends in these regions are at variance with the rest of the world.

Based on length polymorphism, there were 6 C. *hominis* subtypes but the subtyping based on the trinucleotide repeats yielded 10 subtypes, which were different from those, reported elsewhere in Kenya [44]. The subtypes did not show any bias in distribution between the rural or peri-urban areas of residence. These results imply that there are many *Cryptosporidium* subtypes circulating in Kenya some of which could be unique to specific regions of the country.

There were four subtypes identified among the *C. parvum* isolates by use of restriction fragment length polymorphism, but 7 subtypes were identified using the trinucleotide repeat analysis. Most of the *C. parvum* subtypes were isolated from patients residing in rural and peri-urban areas. This is the first report of the *C. parvum* subtypes in Kenya using the CP47 microsatellite gene. Since this genotype of

Cryptosporidium can be transmitted both through the anthroponotic, zoonotic route and even through environmental contamination [45], it is difficult to ascertain the extent to which these modes of transmission are involved in the spread of cryptosporidiosis among HIV positive individuals. This therefore calls for a deeper understanding of the epidemiology of cryptosporidiosis in this region so as to zero in on determinants that govern the distribution of the various genotypes and subtypes. One patient representing 1.1% of all the cryptosporidiosis HIV positive patients, who resided in a rural area, had a mixed infection of *C. parvum* and *C. hominis*, a finding that is consistent with a report from Britain [40]. The presence of mixed infection is an indication of contact with human or animal waste or water that is contaminated with either genotype or both.

This study has revealed that there are more *C. hominis* subtypes that are circulating in the region as compared to *C. parvum* subtypes. There is need, therefore, for a more comprehensive study to look into the *C. hominis* and *C. parvum* populations in terms of the circulating genotypes and subtypes in the North Rift Region of Kenya using more subtyping tools in an endeavor to establish associations of cryptosporidiosis with the various parameters that influence and/or encourage genetic mixing and transmission.

Conclusion

This study established the prevalence among HIV positive patients as 3.2% with *C. hominis* with subtypes being the leading cause of cryptosporidiosis followed by *C. parvum* with seven subtypes and then *C. meleagridis* in that order. It has also established indeed as other studies that mixed *C. hominis* and *C. parvum* constitute about 1% of the cryptosporidiosis burden. The average CD4 count in HIV positive persons with cryptosporidiosis was 76.21 cells per µl, three times lower than in those without crptosporidiosis. In children of five years and below the prevalence was found to be 9.8% whereby *C. hominis* was the leading cause of cryptosporidiosis followed by *C. parvum*. Diarrhea, abdominal pains, CD4 counts and skin rash were significantly associated with cryptosporidiosis among the HIV positive patient and are therefore predictors of cryptosporidiosis.

Piped water and water drawn from wells/boreholes presented a higher risk of infection with *cryptosporidium* while boiling water did not seem protect individuals from *Cryptosporidium* infection.

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