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Diagnostic Procedures, Epidemiology and Genetic Diversity of *Cryptosporidium Species* in Bungoma County, Kenya

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Abstract: ***A prospective study on prevalence of Cryptosporidium and cryptosporidiosis was carried out in Bungoma County, Kenya. A total of 712 fecal samples from children up to five years of age were collected, from four Hospitals during a 30 month period covering January 2011 to June 2013. Overall prevalence of cryptosporidiosis in children was 5.06%. Genotype analysis revealed that C. hominis had the highest prevalence of 38.89% (14/36) of the isolates, 36.11% were C. parvum, while the prevalence of C. meleagridis and C.canis was 16.67% and 5.56% respectively. Cryptosporidiosis was significantly associated with diarrhea (acute/persistent /recurrent) (P=0.01 OR=1.301) and Abdominal swelling and pain (P=0.031, OR=1.56, 95%= 1.04-2.34). The results suggest that prevalence of cryptosporidiosis is comparable to other regions of the world with C. hominis being the most common species circulating in the study area followed by C. parvum and C. meleagridis in that order.***

Keywords: *Cryptosporidium*, Apicomplexa, microscopy, ImmunoCard STAT! Assay, Molecular Methods,

1. Introduction

Cryptosporidium species is a protozoan pathogen of the phylum Apicomplexa that is now recognized as a major cause of Diarrheal disease worldwide [9], [10]. Cryptosporidiosis is typically an acute short term self limiting infection but can become severe and non-resolving in children and immunocompromised individuals such as persons receiving immuno-suppressive drugs and AIDS patients [6]. Since drug therapy for the control or elimination of these organisms is not yet available, persistent infections in these patients are especially severe and can be life threatening.

The potential of *Cryptosporidium* as an opportunistic parasite due to contamination of drinking water supplies indicate that the parasite should be regarded as a major public health problem [9], [19]. To date 16 species of *Cryptosporidium* have been regarded as valid on the basis of host specificity and oocyst morphology [8]. These include *Cryptosporidium parvum* in mammals, *C. hominis*,in humans, *C.meleagridis, C.felis, C.wrairi* in guinea pigs, *C. baileyi* in birds and *C. serpentis* in reptiles . Although the human and cattle genotypes were thought to be the only two genotypes infective for human hosts, it has recently been shown that immuno-compromised and even immunocompetent individuals are more susceptible to more than just these two genotypes of *C. parvum*. Indeed *C.felis, C.meleagridis* and *C. muris* have been associated with human infections [15] [18] [19].

Various methods have been applied to detect oocysts in faeces but the difficulties of discriminating between non-cryptosporidial bodies, acid fast bodies like cryptosporidia and *Cryptosporidium* remain [8]. In the absence of effective therapeutic agents, control and treatment of cryptosporidiosis are dependent upon early and accurate diagnosis and an accurate understanding of the epidemiology and transmission dynamics.

In the present study we compared detection of *Cryptosporidium* species using the ImmunoCard STAT! Assay (Meridian Bioscience Inc. USA) and molecular method using PCR as well as the prevalence of the parasite in the study area.

2. Literature Survey

The protozoan parasite *Cryptosporidium species* is a leading cause of infectious diarrhea in humans and livestock, with fecal-oral transmission by ingestion of oocysts. Infection is generally self-limiting, followed by variable protective immunity involving humoral and cell mediated responses, except in the immune suppressed, when infection may be prolonged and fatal [11]. *Cryptosporidium* oocysts remain viable in water and damp soils, for prolonged periods and are resistant to disinfectants at concentrations usually used in water treatment [2]. Outbreak investigations have shown diverse modes of transmission, including contact with livestock [3], [7], person to person transmission in households and care settings [5], consumption of contaminated foods and drinks, including milk [12], [13]. Cryptosporidiosis is responsible for 8-10% of cases of diarrheal diseases with significant effect on mortality [4], [21] ,[24]. A recent study on cryptosporidiosis conducted in Egypt examined 1275 children, attending two hospitals and found prevalence of 17%. The study also found that children less than 12 months of age were most likely to get cryptosporidiosis and infection was significantly associated with diarrhea, vomiting and a need for hospitalization [25]. A recent survey on the prevalence of cryptosporidiosis among Human Immune Deficiency Virus (HIV) infected and uninfected children with persistent diarrhea at Mulago hospital in Uganda showed 73.6% (67 of 91) of HIV infected children and 5.9% (9 of 152) of HIV negative children were infected [26]. Another recent study on cryptosporidiosis conducted in Kenya examined 4899 samples and showed an overall prevalence of 4%. The

prevalence was highest in children 13-24 months of age (5.2%) and Lowest among those 48-60 months of age [28].

The main symptoms associated with the disease were abdominal pain, vomiting and abdominal swelling [28]. Various methods have been applied to detect oocysts in feces but difficulties of discriminating between non-Cryptosporidial bodies, acid-fast bodies like *Cryptosporidia* and *Cryptosporidium* remain. Screening by use of wet preparations has been found to be insensitive and not particularly helpful, although it may be useful in detecting cysts or ova [1]. A modified Ziehl Neelson staining method has been widely recommended and used. Its main limitation is that it has many stages involving concentration and staining, therefore unsuitable for handling large batches of specimens in routine laboratory examination. Overall, microscopic identification requires trained microscopists and involves time and labor for preparing, staining and examining [11], [14]. As a result, immunoassays for detection of *Cryptosporidium* stool antigen have replaced microscopy as the routine diagnostic procedure of choice in hospitals and public health laboratories that routinely carry out this test [10]. Polymerase chain reaction (PCR) has been used to identify the various species and genotypes/strains of *Cryptosporidium.*

3. Problem Definition

Cryptosporidium species is a common cause of protozoal diarrheal disease in humans especially, children and immuno-compromised patients. There are several species of *Cryptosporidium* that are known to cause both human and animal infections. However, despite this fact being known, not much is known or has been done about the genotypes and epidemiology of this parasite in many parts of Kenya, including the study area. Similarly, patients presenting diarrhea in hospitals are seldom examined for *Cryptosporidium species* despite it being known as one of the major causes of diarrhea. Where the test for *Cryptosporidium species* is specifically requested for (few laboratories offer the test), the procedure is long and time consuming (such as Modified Ziehl Neelson staining) or is expensive (P.C.R procedure). Antigen detection assays have proven utility in the diagnosis of the infection. The assays offer advantages in labor, time and batching efficiency that may lead to reduced infections. The current study was carried out to determine the epidemiology of Cryptosporidiosis, the molecular diversity and the validity of ImmunoCard STAT! Rapid assay as a simpler, less time consuming and economical procedure for diagnosing *Cryptosporidium species.*

4. Materials and Methods

Stool specimens: a total of 712 human fecal specimens from four different sites were collected and preserved in 10% formalin. All the specimens were collected from children up to five years with or without diarrhea attending four hospitals in Bungoma County and had been referred to the laboratories for ova/parasite examination.

Assays:

Fecal concentration and Modified ZN staining
A formalin ethyl acetate concentration was performed on all the specimens before preparing a concentrated wet smear and modified Ziehl Neelson stained smears. In brief stool specimens were passed through a double layered gauze bandage before being centrifuged twice at 400xg for 10 minutes to concentrate. Smears of approximately 20x20mm were examined with x100 oil immersion and the presence or absence of *Cryptosporidium* was recorded.

ImmunoCard Assay
The ImmunoCard STAT! Crypto /Giardia rapid assay was performed on un-concentrated formalin fixed stool specimens as specified by the Manufacturer (Meridian Bioscience Inc USA). Results were viewed after 10 minutes. A positive control line was visible on the device each time a test was completed. A positive reaction appeared as a grey black band visible at the *Cryptosporidium/Giardia* area in the test window. No reaction in the test window and a positive control line was interpreted as a negative result. Tests on samples with weak or faint reactions using the ImmunoCard assay were repeated.

PCR analysis
Those samples that were found to be positive for *Cryptosporidium by* microscopy were further processed for molecular genotyping as described earlier [24]. In brief, the samples were preserved in 75% ethanol. Approximately 200µLof fecal suspension was then washed three times in distilled water before DNA extraction. Genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). Supernatants containing DNA were stored at −20°C until use. Nested PCR was used to amplify an approximately 840 base pair (bp) long fragment corresponding to the *Cryptosporidium* 18S rRNA gene using two sets of oligonucleotide primers: 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3'forprimary PCR and 5'GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-CTCATAAGGTGCTGAAGGAGTA-3' for secondary PCR. The PCR reactions were carried out in 25 µl volumes containing 12.5 µl DreamTaq Master Mix (Promega, Madison, WI), 0.5 µl of each primer (10 µM), 6.5 µl nuclease-free water (Promega) and 5 µl DNA template. For amplification, templates were subjected to a hot start at 95°C for 15 min followed by 35 cycles of 94°C for 45 s, 53°C for 75 s and 72°C for 1 min followed by a final extension at 72°C for 7 minutes. The PCR products were stored indefinitely at 4°C. Secondary PCR products were analyzed by 2% agarose gel electrophoresis and Ethidium bromide staining

Data entry, handling and analysis
The data was manually entered using SPSS data editor version 17.0 (Microsoft inc. USA) and analyzed using SPSS version 11.5 (Microsoft Inc. USA). To determine associations between infection with cryptosporidiosis, diarrhea or other symptoms, and risk factors associated with it, a Pearson's Chi square test was used to compare samples. A P value of < 0.05 was considered statistically significant.

Ethical considerations

The study was reviewed and approved by the National Council for Science and Technology and Innovation (NACOSTI) of Kenya(Research permit No. NCST/RRI/12/1/MED/223). All parents/ guardians were informed of the purpose of the study and voluntary consent was sought before inclusion into the study. Results for *Cryptosporidium* and other intestinal parasites were handed to the caring hospital staff for appropriate action.

5. Results

Prevalence of *Cryptosporidium*

Out the 712 samples collected, 36 of them were found to be *Cryptosporidium* positive, translating to an overall prevalence of 5.06%. Chwele Hospital had the highest prevalence of 6.51% (11/169) while Webuye District Hospital had the least prevalence of 3.74% (7/187). However there was no significant difference in *Cryptosporidium* prevalence in four hospitals (*P=0.0563).*

Table I: prevalence of cryptosporidium species by centres

Hospital	*Total samples*	*Cryptosporidium cases*	*% prevalence*
Bungoma	193	08	4.15
Chwele	169	11	6.51
Kimilili	163	10	3.74
Webuye	187	07	5.05
Total	712	36	5.06

***Cryptosporidium* distribution by age**

Prevalence of *Cryptosporidium* was highest in children aged between 13-36 months with overall prevalence of 5.83% (21/36) and least in those aged 37-60 months at 1.67% (6/36). There was a significant association between age and *Cryptosporidium* infection (*P=0.0345) (T*able II)

Table II: *Cryptosporidium spp* distribution by Age

Age range (Months)	Total patients	*Crypto* case	% prevalence in age range	OR	95%ci	P value
1-12	181	09	4.97	1.0	-	-
13-24	185	13	7.02	1.49	1.06-2.09	0.023*
25-36	132	08	6.06	0.54	0.33-0.87	0.011*
37-48	113	04	3.53	0.23	0.53-0.98	0.06
49-60	101	02	1.98	0.38	0.16-0.89	0.08
Total	**712**	**36**	**5.06**			

***Significant**

Prevalence by seasons.

Prevalence of *Cryptosporidium* was highest during the months of June- July and October –November, which coincided with the tail end of the long and short rains respectively (figure I). Univariate analysis showed that cryptosporidiosis was seasonal with more infections likely to occur during the wet season [(OR-1.73, CI=1.23-241) *P<0.001*]

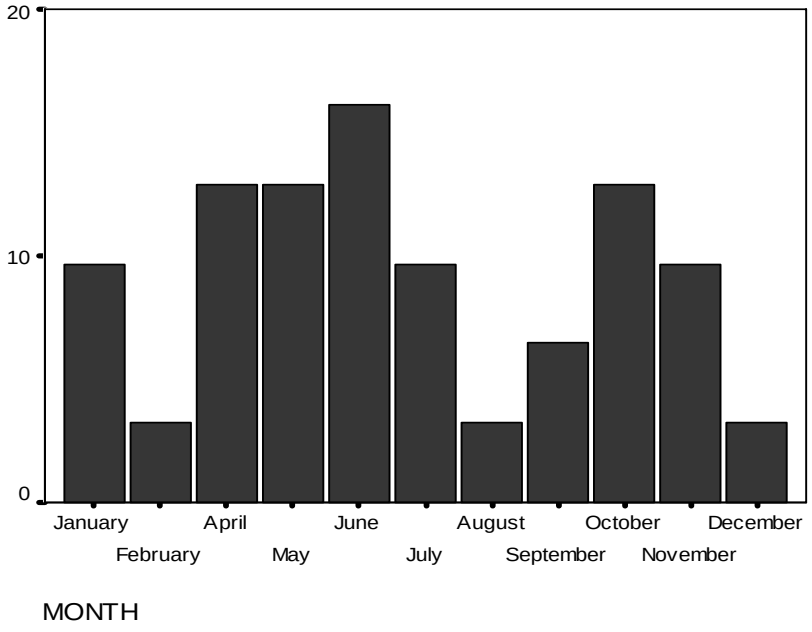

Figure 6: Distribution of *Cryptosporidium spp* by seasons. There was peak prevalence in June and October that coincided with ending of the long and short rains in the study area.

Symptoms associated with Cryptosporidium infections.

Diarrhea was the most common symptom in *Cryptosporidium* infected patients [39.99%], followed by abdominal pain/swelling/stomach muscle cramps [33.33%]. The least symptom shown by patients was vomiting at 4.12% Cryptosporidiosis was significantly associated with diarrhea (acute/persistent /recurrent) (*P*=0.01 OR=1.301) and Abdominal swelling and pain (*P*=0.031, OR=1.56, 95%= 1.04-2.34). There was no significant association of cryptosporidiosis with vomiting (*P*=0.334, OR=0.831, 95%CI= 0.657- 1.23). (table III)

Table 3: Symptoms associated with *Cryptosporidium* infections

Symptom	Crypto present	Crypto absent	Total	Overall percentage	X^2	P-value
Diarrhea	14 (38.9%)	254	268	5.22%	0.48	≤0.0010

Abdominal swelling/pain	12 (31.6%)	213	225	5.13%	0.44	0.05
Dehydration	6 (16.7%)	116	122	4.92%	3.8	0.063
Vomiting/others	4 (1.11%)	93	97	4.12%	6.9	0.79
	36	676	712	5.06%		

Occurrence and molecular diversity of *Cryptosporidium species*

PCR analysis that targeted the conservative 18S Ribosomal RNA for amplification. *Cryptosporidium hominis* had the highest prevalence of 38.9% (14/36), followed by *C. parvum* at 36.11% (9/36), *C. meleagridis* 16.67% (6/36) and *C. canis* at 5.56 % (2/36). One sample was not amplified and therefore could not be identified.

Table 6: Cryptosporidium genotypes

Genotype	Total	Prevalence %
Cryptosporidium hominis	14	38.89
Cryptosporidium parvum	13	36.11
Cryptosporidium meleagridis	6	16.67
Cryptosporidium canis	2	5.56
Unknown genotype	1	2.78

Validity of ImmunoCard Stat! Assay

The detection of *Cryptosporidium* by PCR was compared with detection using the Immuno card Assay! (Meridian Bioscience Inc. USA). The assay only detected *C. parvum* and not the other genotypes. The results showed the Assay had sensitivity and specificity of 92.8% and 90.5% respectively

Table 5: Detection of *Cryptosporidium parvum* by ImmunoCard Stat! Assay

PCR DETECTION		IMMUNOCARD STAT! ASSAY	
		Positive	Negative
	Positive	13	2
	Negative	1	19
		14	21

6. Discussion

This study focused on the current state of *Cryptosporidium* infections in children up to five years of age, who presented gastroenteritis to the four participating hospitals and were referred to the laboratories for ova and cyst examination.

This study is unlike other studies that have focused on the high risk groups such as children with HIV and/or those with persistent diarrhea only or day care centres. The results showed an overall *Cryptosporidium* prevalence of 5.06%. This was slightly higher than results from previous studies in the country. For example, a 2006 study in Nairobi, Kenya focusing on the same age range showed an overall prevalence of 4% (Wangeci *et al., 2006*). A higher prevalence than 5.06% has also been previously reported in patients whose immunity was impaired, for instance in HIV infected children in Mulago Hospital, Uganda where the prevalence was 73.6% [26] and 17% in a study in Egypt [25]

Cryptosporidium infections were significantly found to be seasonal with peaks and lows. The highest prevalences were in the months of June and October which marked the tail ends of the long and short rains respectively in the study area. This seasonality in infection was also found in similar studies [27], [28]. This peak prevalence during the end of the rain season coincided with results of two other studies carried out in Malawi and Kolkata, India, which also had peak prevalence during the rainy season between March and October [22], [27]. However the peak prevalence in this study differed from the peaks in other studies conducted in the country, which coincided with the dry season of November- February [28].

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The ability to simultaneously detect and distinguish between *Giardia* and *Cryptosporidium* antigens in fixed and unfixed stool specimens using the ImmunoCard STAT! Assay is a novel idea. The detection of *Cryptosporidium parvum* by PCR was compared with detection by ImmunoCard STAT! Assay! For detection of *Cryptosporidium parvum*, the sensitivity and specificity were 92.8% and90.5% respectively. A study by Schuurman *et al.*, [29], found sensitivity and specificity of 98-100% and $\geq$ 97% respectively for detection of both parasites. In another study to evaluate three commercial assays for detection of *Giardia* and *Cryptosporidium parvum*, the ImmunoCard Assay was found to have sensitivities of 81% and 78% respectively, and specificities of above 96% in both cases (Johnson *et al.*, 2003) However the sensitivity and specificity of the method as reported in this study are lower than other immunoassays such as the Direct Fluorescent Antibody (DFA) tests that detect intact organisms and Enzyme Immunoassay (EIA) tests which detect stool antigens [20], [23]

7. Conclusion

Based on the results it is concluded that *Cryptosporidium spp* is one of the most important parasites that cause significant morbidity in children and is one of etiological agents responsible for child hood watery diarrhea and abdominal pain in Kenya .*Cryptosporidium spp* infections are marked with seasonality with peaks coinciding with the end of the rain seasons possible due contamination of drinking water by flood waters carrying animal and human wastes Cryptosporidiosis is attributed to several species of the parasite four, of which were identified in the study area. Transmission was mainly anthropogenic especially with the 'human genotypes' such as *Cryptosporidium hominis*. Because it had sensitivities and specificities of above 90% for detection of both parasites, it was concluded that the

ImmunoCard Stat! Assay is a valid diagnostic kit for rapid detection of *Cryptosporidium parvum*

8. Future Scope

Although a lot of research has been carried out about *Cryptosporidium species*, there remain a number of issues that are yet to be resolved.

Number of Cryptosporidium species
There has been an explosion of descriptions of new species of *Cryptosporidium* during the last two decades. This has been accompanied by confusion regarding the criteria for species designation, largely because of lack of distinct morphological differences and strict host specificity among *Cryptosporidium species.* There is therefore need for clarification of *Cryptosporidium* taxonomy which will be useful for understanding the biology of *Cryptosporidium species*, assessing the public health significance of *Cryptosporidium species* in animals and the environment, characterizing transmission dynamics, and tracking infection and contamination sources.

Transmission of Cryptosporidium species
One major problem in understanding the transmission of *Cryptosporidium* infection is the lack of morphologic features that clearly differentiate one *Cryptosporidium* species from many others. Hence, one cannot be sure which *Cryptosporidium species* is involved when one examines oocysts in clinical specimens under a microscope. Associated with the problems in taxonomy and nomenclature is the public health importance of various *Cryptosporidium species*. Without clear diagnostic features that allow the differentiation of *Cryptosporidium species*, we do not know the precise number of species infecting humans, the burden of disease (sporadic and outbreak related) attributable to different species or strains/genotypes, and the role of species and strains/genotypes in virulence or transmission in humans. These questions present challenges to our understanding of the epidemiology of cryptosporidiosis. Revision of *Cryptosporidium* taxonomy, therefore, is useful to our understanding of the biology, epidemiology and public health importance of various *Cryptosporidium species*

Prevalence of *Cryptosporidium*
It is not known exactly how many cases of *Cryptosporidium* infections actually occur annually in many regions. Many people do not seek medical attention or are not tested for this parasite and so *Cryptosporidium* often goes undetected as the cause of intestinal illness. Health professionals in some countries are on the lookout for cases of *Cryptosporidium* through surveillance programs at hospitals clinics and laboratories. Although oocysts are present in most surface waters (lakes and rivers) many of which supply public drinking water, only few laboratories have specialized capabilities to detect the presence of *Cryptosporidium*. Again current sampling methods are unreliable as it is difficult to recover oocysts trapped on the material used to filter water samples.

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