

## Incidence of cryptosporidiosis species in paediatric patients in Malawi

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### SUMMARY

We determined the incidence of cryptosporidiosis in children aged <5 years presenting with diarrhoea in an urban and rural hospital-based setting in Malawi. Stools were collected over a 22-month period during both rainy and dry seasons. A range of microscopic methods were used to determine the presence of *Cryptosporidium* spp. oocysts. Species determination was by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of oocyst-extracted DNA using 18S rRNA and COWP gene loci. *Cryptosporidium* spp. oocysts were seen in 5.9% (50/848) of samples, of which 43 amplified by PCR–RFLP indicated the following species: *C. hominis*, *C. parvum*, *C. hominis/C. parvum*, *C. meleagridis* and *C. andersoni*. Seven samples could not be amplified by PCR. Wider species diversity was found in the rural setting, and may be a result of increased malnutrition and zoonotic exposure in this area. Improvements in water, sanitation, household hygiene and animal control are required to reduce the incidence of infection in this population.

### INTRODUCTION

Diarrhoeal diseases in children aged <5 years are a significant cause of morbidity and mortality in the developing world [1], and in Malawi, they are thought to be the sixth leading cause of mortality which currently stands at 182/1000 live births [2]. Cryptosporidiosis is an acute, self-limiting diarrhoeal illness in immunocompetent patients, but in the immunocompromised, especially those with HIV/AIDS, and the malnourished, the disease can be severe, protracted, debilitating, and may lead to death. In Africa, cryptosporidiosis has been associated with

3.8–26% of all diarrhoeal illness in the paediatric population [3–5], and has been diagnosed in at least 47% of African countries. In Malawi, up to 10% of diarrhoea in children aged <5 years is attributable to *Cryptosporidium* spp. infection with up to 21% reported in the immunocompromised population [6; M. Perez, unpublished observations]. Of the 14 presently accepted ‘valid’ species of *Cryptosporidium*, seven have been found to infect humans to date [7, 8]. In sub-Saharan Africa alone, four of these species have been identified as causing infection to date, namely *C. hominis*, *C. parvum*, *C. meleagridis* and *C. muris* [9–13].

In 2003, it was estimated that at least 14% of the population in Malawi were infected with HIV, and that 67% of preschool children in Malawi are chronically malnourished causing many children to

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fail to reach their full growth and cognitive potentials in later life [14]. In the absence of inexpensive, well tested chemotherapy for cryptosporidiosis, prevention of transmission is the only effective means of reducing the incidence of disease and its subsequent impact on morbidity and mortality [15].

To reduce exposure to infectious *Cryptosporidium* oocysts in the young and immuno-compromised, both risk factors and the epidemiology of disease must be understood. As all species do not appear to cause infection in humans, and the majority of human infective species cannot be differentiated microscopically because of overlap in their oocyst dimensions, both microscopic and molecular methods, which can discriminate between all valid *Cryptosporidium* spp., are required to identify potential sources of infection.

Here we present data, accrued over 2 years, of the incidence of *Cryptosporidium* spp. in children aged <5 years in Malawi, using conventional microscopic and molecular species typing tools. This was part of a larger study which investigated the risk factors associated with childhood cryptosporidiosis and the environmental sources of *Cryptosporidium* in Malawi between urban and rural areas.

## MATERIALS AND METHODS

### Sample procurement

We studied two districts of the southern region of Malawi representing both urban (Blantyre) and rural (Chikwawa) settings. Permission to conduct the study was obtained from the National Health Sciences Research Committee (Ministry of Health and Population, Malawi Government). The study was conducted from the main Government hospitals in both areas, and local health clinics.

Stool samples were collected from children aged <5 years with diarrhoea. Diarrhoea was defined as an increase in the frequency and/or change in the consistency (to loose or watery) of the stool as determined by the child's mother/guardian. Samples were procured by medical staff who requested that guardians supply a stool sample from the child in a 30 ml plastic container (Enterprise Containers, Blantyre, Malawi). Guardians who provided baseline information; received advice on reducing and treating diarrhoea and were provided with soap for hand washing, oral rehydration salts for (ORS) treatment. Stool samples were transported in a cool box and stored in the laboratory at 4 °C.

## Detection methods

### Microscopy

*Cryptosporidium* oocysts were detected in direct faecal smears by the modified Ziehl–Neelsen (mZN) and auramine phenol (AP) methods [16]. Sub-samples of all stools were stored without preservative at 4 °C and subjected to further microscopic and molecular analyses in the United Kingdom (Scottish Parasite Diagnostic Laboratory). Putative mZN and/or AP positive samples were confirmed by immunofluorescence (IF) with a commercially available fluorescein isothiocyanate-labelled monoclonal antibody reactive with exposed epitopes on *Cryptosporidium* spp. oocysts (FITC-C-mAbs) (Cryptoglo, Waterborne Inc. New Orleans, USA; CryptoCel, TCS Water Sciences, Australia), and the nuclear fluorogen 4'6-diamidino-2-phenyl indole (DAPI) [17]. Positive samples, viewed at ×400, were scored from negative to +3 [18]. Nomarski differential interference contrast (DIC) microscopy was used to determine internal morphology and fluorescence at ×1000 magnification using an Olympus BH-2 fluorescence microscope equipped with DIC optics. A blue filter (480 nm excitation, 520 nm emission) was used to detect FITC-C-mAb-labelled oocysts and DAPI was detected using a UV filter block (350 nm excitation, 450 nm emission).

### Molecular typing

*Oocyst concentration and DNA extraction.* Oocyst positive and putative positive faecal samples were purified by a modified water–ether concentration method [19, 20]. With semi-solid samples, a pea-sized faecal sample was diluted in 200 µl distilled water and emulsified in a 1.5 ml microcentrifuge tube, and for liquid faeces, 200 µl were transferred to a 1.5 ml microcentrifuge tube. Samples were concentrated as previously described [19, 20]. Finally the supernatant was aspirated down to 100 µl, the remaining sample resuspended and 5 µl transferred to a well slide for subsequent FITC and DAPI staining as described above. The remainder of the sample was washed twice, as described above, with lysis buffer [LB; 50 mM Tris–HCl (pH 8), 1 mM EDTA (pH 8), 0.5% SDS].

Where insufficient faecal sample was available or where PCR amplification was inhibited, oocysts were recovered from IF slides which had been prepared at the time of sample procurement. Briefly, coverslips were carefully removed from slides to

expose the stained sample. Then, 20  $\mu$ l LB was added and the dried sample mixed with LB. The resuspended sample was transferred into a micro-centrifuge tube and sample mixing was repeated five times to ensure maximum oocyst recovery from the sample well. Where necessary to maximize oocyst recovery, sample volume was increased to 100  $\mu$ l with LB [21].

DNA was released from oocysts by freeze-thawing and proteinase K digestion [22]. The supernatant (70  $\mu$ l), containing released DNA, was transferred to a clean (DNase-/RNase-free) tube and either used immediately for PCR amplification or stored at  $-20^{\circ}\text{C}$  until used.

#### PCR protocols

*Cryptosporidium* spp. identity was determined for all samples using the following PCR–RFLP assays:

- (a) direct PCR amplification of the 18S rRNA gene locus [23], co-amplification with an internal control [24] followed by simultaneous digestion with *DraI* and *AseI* (D18S rRNA) [21];
- (b) nested PCR amplification of the 18S rRNA gene locus [21, 23] followed by simultaneous digestion with *DraI* and *AseI* (N18S rRNA);
- (c) single tube nested PCR amplification of the *Cryptosporidium* oocyst wall protein (STN-COWP) gene locus, followed by digestion with *TaqI* [25].

In addition, when samples failed to amplify, or gave insufficient amplicon for digestion using PCR (c), samples were also subjected to two-step nested (2SN) PCR at the Homan *et al.* [25] COWP gene locus (N-COWP) or the nested 18S rRNA PCR [26].

All PCR reactions were performed in a GeneAmp<sup>®</sup> Model 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) using thin-walled 0.5 ml DNase-/RNase-free PCR tubes. PCRs (a) and (b) (18S rRNA gene locus) generate an amplicon of 435 bp. Endonuclease restriction with *AseI* and *DraI* produces the following fragments in order by size as they are separated on the gel: *C. hominis*: 222, 104, 112 bp; *C. parvum*: 219, 104, 112 bp; *C. meleagridis*: 171, 104, 112, 47 bp; *C. felis*: 189, 112, 104, 50 bp and *C. muris*: 320, 112 bp. PCR (c) generates an amplicon of 640 bp. Endonuclease restriction with *TaqI* produces the following fragments: *C. hominis*: 470, 170 bp; *C. parvum*: 374, 266 bp.

Table 1. Results of microscopy and PCR-positive tests for presence of *Cryptosporidium* sp.

Sampling area	AP				
	mZN	positive	IF*	PCR	Total
Rural	16	19	13	18	25
Urban	15	21	23	25	25
Total	31	40	36	43	50

mZN, Modified Ziehl–Neelsen; AP, auramine phenol; IF, immunofluorescence; PCR, polymerase chain reaction.

\*  $\chi^2 = 10$ , D.F. = 1,  $P < 0.005$ .

## RESULTS

Eight hundred and forty-eight diarrhoeal stool samples (urban  $n = 425$ , rural  $n = 423$ ) were collected between February 2001 and December 2002, of which 50 samples (rural  $n = 25$ , urban  $n = 25$ ) were confirmed *Cryptosporidium* positive by a combination of microscopy and molecular techniques. The efficiency of detection of the various techniques used in this study is shown in Table 1.

The species of *Cryptosporidium* infecting these children are shown in Table 2. *C. hominis* accounted for 48% of positive samples and *C. parvum* for 18%. Four samples generated amplicons typical in size and RFLP profiles to that of *C. hominis/C. parvum* with N18S rRNA. However, further PCR targeting the COWP gene loci and the N18S rRNA locus of Xiao *et al.* [26] failed to generate positive PCRs for these four samples. A further sample generated an amplicon typical in size to that of *C. andersoni/C. muris* and a typical *C. andersoni* RFLP profile with *DdeI*, but insufficient sample was available for confirmation. Seven samples did not generate a PCR product despite containing oocysts. Two of these samples were shown to be inhibitory with the use of the internal control. However, no inhibition was evident in the remaining five samples, which were all positive when direct smears were examined by at least two microscopic methods. Examination of the water–ether concentrate failed to identify oocysts in four of these, and the remaining sample was found to have a number of empty shells with no nuclei evident when examined with DAPI.

No significant difference in the number of cases infected with different species was seen between the rural and urban areas ( $\chi^2 = 0.012$ , 1 D.F.,  $P > 0.5$ ) however, the range of species present in Chikwawa was more diverse. Both rural and urban cases were

Table 2. *Cryptosporidium* spp. infecting Malawian rural and urban <5-year-olds

Sampling area	Species of <i>Cryptosporidium</i>						No amplicon	Total
	<i>C. hominis</i>	<i>C. parvum</i>	<i>C. hominis</i> / <i>C. parvum</i> (mixed)	<i>C. hominis</i> / <i>C. parvum</i> (untyped)	<i>C. meleagridis</i>	<i>C. andersoni</i>		
Rural	9	3	0	3	2	1	6	25
Urban	16	7	1	1	0	0	1	25
Total	25	10	1	4	2	1	7	50

Table 3. Sensitivity of molecular methods for detecting *Cryptosporidium* species in stools from Malawian rural and urban <5-year-olds

Species identified	PCR-RFLP								
	D18S rRNA + internal control	N18S rRNA	RFLP <i>AseI</i> & <i>DraI</i>	RFLP <i>DdeI</i>	STN-COWP	N-COWP	RFLP <i>TaqI</i>	N-Xiao	RFLP <i>SspI</i> / <i>AseI</i>
<i>C. hominis</i>	15/25*	25/25	25/25	n.a.	11/25	12/16	21/21	4/4	4/4
<i>C. parvum</i>	7/10	10/10	9/10†	n.a.	6/10	9/10	9/9	1/1	1/1
<i>C. parvum</i> and <i>C. hominis</i>	1/1	1/1	1/1	n.a.	1/1	1/1	1/1	n.a.	n.a.
? <i>C. parvum</i> / <i>C. hominis</i>	1/4*	4/4	3/3	n.a.	0/4	0/4	0/4	0/4	n.a.
<i>C. meleagridis</i>	0/2*	2/2	2/2	n.a.	0/2	0/2	n.a.	0/2	n.a.
<i>C. muris</i> / <i>C. andersoni</i>	0/1	1/1	1/1	1/1	0/1	0/1	n.a.	0/1	n.a.

PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; STN-COWP, single tube nested PCR amplification of the *Cryptosporidium* oocyst wall protein; N-COWP, nested PCR amplification of the *Cryptosporidium* oocyst wall protein; N-Xiao, nested PCR amplification of 18S rRNA gene loci as described by Xiao *et al.* [26]; n.a., not applicable.

\* In each case one sample which appeared negative was shown to be inhibitory by the use of the internal control.

† Concentration of PCR product too low to produce visible RFLP pattern.

identified primarily in the Malawian rainy season (rainy season 8.1% vs. dry season 3.7%,  $\chi^2=6.9$ , 1 D.F.,  $P<0.01$ ). The median age of cases was 11 months (IQR=9.5) and the median age of participants was 10 months (IQR=11). Eighty-five percent of cases were aged <2 years, and all cases were aged <4 years. Fifty-two percent of cases were between 7 and 12 months old indicating a significantly higher number of cases in that age group ( $\chi^2=22.1$ , 7 D.F.,  $P<0.005$ ). Age was not an influencing factor with regard to species distribution in cases. With the exception that both our *C. meleagridis* cases were female, gender was not significantly associated with either cryptosporidiosis or specific *Cryptosporidium* spp. causing infection. Due to the low numbers of *C. meleagridis* cases found in this study gender is not thought to be significant.

The sensitivity of the molecular methods used for detecting *Cryptosporidium* spp. is summarized in

Table 3. N18S rRNA proved to be the most consistent, confirming 100% of cases. The STN-COWP and the N-COWP assays did not amplify DNA as consistently as the N18S rRNA gene locus assay [22], however, it discriminates between *C. hominis* and *C. parvum*. The N18S rRNA gene assay of Xiao *et al.* [26] can differentiate between *C. parvum* and *C. hominis*, however, it is less sensitive than the N18S rRNA [22] and failed to amplify the four samples that were negative by the STN-COWP (Table 3). Species identity was not possible with four samples using this COWP gene locus.

## DISCUSSION

Very few studies have been conducted in sub-Saharan Africa to assess the species of *Cryptosporidium* present in a population. Previously, microscopical studies for diagnosing cryptosporidiosis in developing countries

have assumed that the infecting species were *C. parvum* and *C. hominis*. More recently, studies in Kenya, Uganda and Malawi have assessed the species types and have identified *C. parvum*, *C. hominis*, *C. muris* and *C. meleagridis* [10–13]. Despite an insufficient difference in oocyst size and morphology to be able to differentiate the species, this study has shown that three different species were present in children aged <5 years. Each species has different implications for the source of infection, subsequent risk assessment and epidemiology. It is interesting to note that *C. hominis* is mostly associated with outbreaks of cryptosporidiosis in developed countries with *C. parvum* being the main cause of sporadic cases [18]. However, in this and other studies in sub-Saharan Africa, the dominant species has consistently been *C. hominis* in sporadic cases thereby indicating the increased potential for anthroponotic transmission in this environment.

### Microscopic examination

mZN generated the lowest number of oocyst-positive samples, and, in this study, led to misdiagnosis and under-diagnosis of infection. The number of positive samples diagnosed with mZN increased as oocyst density increased (44% detection with 0/1+ sample, 100% detection with 3+ samples) as previously described [27–29]. AP and IF were better at detecting oocyst-positive samples, but neither can be relied on solely for maximizing the detection threshold. The hierarchy for detecting oocysts tinctorially on slides was AP > IF > mZN (Table 1).

Oocysts in 14 samples [*C. hominis* ( $n=3$ ), *C. parvum* ( $n=1$ ), *C. hominis/parvum* ( $n=1$ ), *C. andersoni* ( $n=1$ ), *C. meleagridis* ( $n=1$ ), no PCR amplification ( $n=7$ )] failed to stain with the two FITC-C-mAbs used when labelled on direct smears (un-purified) and after water–ether purification (semi-purified), but *Cryptosporidium* oocysts/DNA were detected by at least two other methods (mZN, AP, DIC and PCR–RFLP). A significant number of these occurred in samples procured in the rural area ( $\chi^2=10$ , 1 D.F.,  $P<0.005$ ) where a minority of samples may have been subject to temperature fluctuations after deposition and storage prior to collection (maximum 3 days). Not all *C. parvum* and *C. hominis* isolates can be stained with any of the commercially available FITC-C-mAbs (R.A.B. Nichols, unpublished observations) and it is likely that these oocysts did not react with the commercial FITC-C-mAbs used in this study.

### Molecular techniques

The majority of samples (81%) contained *C. hominis* or *C. parvum* oocysts. *C. meleagridis* was detected in two samples, and is the first description of human infection with *C. meleagridis* in Malawi. The *C. andersoni* 18S rDNA amplicon [24] was detected in one sample, however, insufficient material was available for confirmation. Human infection with *C. muris* has been reported from Kenya [30]. *C. andersoni* has not been previously reported in a human case, however, a putative *C. muris*-like sample reported in an HIV+ patient in France [31], had greater sequence similarity to *C. andersoni* than to *C. muris* [32].

Ten oocyst-positive stool samples were lost in transit between Malawi and the SPDL, but the *Cryptosporidium* spp. infecting these ten cases were identified from oocyst-positive slides, processed for immunofluorescence, and obtained at the time of sample procurement in Malawi. Oocyst density ranged from  $\pm$  to 3+ and the *Cryptosporidium* spp. present were detected in all 10 samples using N18S rRNA. A further seven samples, where PCR amplification was unsatisfactory, were assessed using similarly fixed slides and six of these yielded positive results. The overall positivity of PCR–RFLP for species identity from stained slides was 94%. This is similar to that of Amar and colleagues [33, 34] who demonstrated an overall sensitivity of 90%, however, in contrast to these workers who stored their samples on slides for up to 8 weeks at room temperature prior to testing, our slide samples were prepared 2 years previously and stored at 15–30 °C. Given our problems with sample storage and exposure to temperature fluctuations, we recommend further investigations into the use of oocyst-positive methanol fixed slide samples for species/genotype/subtype identification by PCR, particularly with respect to studies in developing countries where facilities can be limited.

Seven samples did not produce amplicons by PCR, and had no fixed slides from the time of procurement available for further testing. Two samples were shown to be affected by inhibition through the use of an internal control, amplifiable with the same primers [24]. Inhibition in PCR may be due to a number of factors, not least the diet of the case and effect of polysaccharides associated with consumption of plant material [35]. The diet and age of children in which inhibition was noted did

not indicate a particular trend in this study (data not shown). No preservatives were used in samples so their inhibitory influence can be ruled out. Microscopic examination by FITC-C-mAbs, DAPI and DIC of the 5  $\mu$ l of concentrated sample showed that one sample had a number of oocysts, but these were empty, indicating a dearth of DNA to amplify by PCR. Oocysts were not found in the remaining four samples when examined by FITC-C-mAbs, DAPI or DIC. Loss of sporozoite DNA might have occurred during long-term storage without preservative [23]. The effect of this loss may also have been compounded by the ability of the water-ether concentration technique to concentrate oocysts causing shells and DNA to be lost to waste. Supernatant recovered from samples prior to water-ether concentration and stored at 4 °C was subjected to freeze-thaw extraction and PCR amplification for these four samples failed to yield a positive result.

PCR techniques used differed in their efficiency to amplify target DNA, and amplification of the 18S rRNA gene using a two-step method [21, 23] was the only method found to give 100% positive results for all samples where amplification was anticipated. The inefficiency of the direct compared to the 2SN18S rRNA method was also noted by Nichols *et al.* [21]. Amplification of oocyst DNA using the single tube nested PCR for the COWP gene loci was found to be variable with an overall success rate of 47.5% for *C. parvum* or *C. hominis* isolates [25]. There was an 80% correlation between samples which failed to produce an amplicon by the single tube nested method targeting the COWP gene loci and the direct 18S rRNA gene loci method, of which only three samples were shown to have any inhibition. Samples which failed to amplify, or produced an inadequate quantity of amplicon for digestion by STN-COWP, were subjected to a 2SN method targeting the same COWP gene loci. Of these 16 samples, 71% produced an amplicon. Therefore, an overall 80% positive result was achieved in samples containing *C. parvum* or *C. hominis* when targeting the COWP gene compared to an overall 100% positive result when using the 2SN method targeting the 18S rRNA gene. The results of this study therefore advocate targeting 18S rRNA which allowed the identification of all species of *Cryptosporidium* to be followed by a 2SN-PCR targeting the COWP gene to differentiate between *C. parvum* and *C. hominis* where necessary.

### Urban vs. rural distribution

No studies have previously assessed the difference between rural and urban infection rates, and species diversity of cryptosporidiosis in sub-Saharan Africa. It was anticipated that a higher proportion of zoonotic infections would be present in the rural area due to the free-range nature of farming, and the high occurrence of animal faeces in and around homes. We do know that *C. parvum* oocysts are present in animal stools in this area (Z. Banda, unpublished observations). However, interestingly, we found the majority of *C. parvum* cases in our urban setting (70%), where animals are not as likely to be present in close proximity to the home. As *C. parvum* can also be transmitted from person to person, we cannot exclude the possibility that these infections may have been of human origin. A recent study by Peng *et al.* [13] identified *C. hominis* and *C. parvum* as the species causing cryptosporidiosis in Blantyre paediatric patients. In addition they indicated that *C. hominis* was the highest cause of cryptosporidiosis (95%). Our study indicates that although *C. hominis* was of higher occurrence in the same sample area (64%), *C. parvum* also played a significant part by causing 36% of infections in the urban setting.

Although the level of *C. parvum* was not higher than the *C. hominis* infection as may have been expected, it was interesting to note the increased diversity of species in cases in the rural setting. Both cases of *C. meleagridis* were found in this area in female patients aged between 6 and 12 months old. Epidemiological data collected during home visits to these cases did not indicate any risk factors different to those of other cases included in the study (data not shown), therefore it is not possible to determine the source of infection at this time. *C. meleagridis* has been associated with both immunocompetent and immunosuppressed cases [11, 30, 36]. However, the proportion of positive cases in this study is higher than that estimated in the developed world (1%) [37] at 4% which may mean that the infection is more common in this area due to immunosuppression attributable to the high levels of malnutrition and HIV in this population. As *C. meleagridis* was only identified in the rural population it may also signify the greater risk of infection due to closer contact with livestock which live within, and in close proximity to households. Animal stools sampled in close proximity to the home of cases, and examined by microscopy failed to identify *Cryptosporidium*

oocysts and could, therefore, not be attributed as the cause of infection (data not shown).

### Seasonal distribution

A relationship is evident between the presence of *Cryptosporidium* spp. and annual rainfall with 70% of cases occurring during the rainy season.

Previous studies have shown correlations between the commencement of the rainy season and an increase in cases of *Cryptosporidium* spp. in sub-Saharan African countries [38, 39]. Although few studies have identified specific sources of infection for cases, this increase may be attributed to a number of factors which occur in the rainy season. First, oocysts present in both animal- and human-derived faeces may be washed from surrounding areas into drinking water sources. In Malawi, this is compounded by the use of unprotected water sources in the rainy season (Chikwawa 40%, Blantyre 18%), including rivers and unprotected wells, in conjunction with the lack of sanitary facilities (Chikwawa 60%, Blantyre 35%, without sanitary facilities) [2]. In addition, an increase in surface water levels, which provide a source for animals to drink from and children to play in, should also be considered. In high density housing areas (urban), both the close proximity of houses and the lack sanitary facilities may contribute to the rapid spread of infection through poor hygiene and human waste disposal practices. This is supported by the higher number of *C. hominis* infections, which are primarily associated with person-to-person transmission, in the urban area. Rural incidence between the seasons was not found to be significantly different, compared to that of the urban area. This may be attributed to the higher level of poor sanitation and unprotected water sources in this district, in conjunction with the increased potential for zoonotic spread of infection.

### Age distribution

The majority of cases occurred in the 0–24 months age group (88%), and may, in part, reflect the high percentage of children from this age group that were sampled (79%), which may in turn reflect the vulnerability of children in this age group to contract diarrhoeal illness. Fifty-two percent of cases occurred in children aged between 7 and 12 months. Results indicate that there is a significantly higher risk of contracting cryptosporidiosis in that age group

( $\chi^2=22.1$ , 7 D.F.,  $P<0.005$ ). The incidence of all species of *Cryptosporidium* identified in this study were higher in this age group than any other (*C. hominis* 40%, *C. parvum* 30%, *C. meleagridis* 100%, mixed 100%, *C. parvum/C. hominis* 50%). This general trend was also noted in a previous Kenyan study [40], and may be attributed to the reduction in assisted immunity from breastfeeding at that age. Partial immunity from previous exposure may then be protecting the higher age groups from an equivalent rate of infection. This is a phenomenon that requires further seroepidemiological investigation and should be the subject of a further cohort study.

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### DECLARATION OF INTEREST

None.

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