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Epidemiology of diarrhoeal disease in rural Malawi – a case study of cryptosporidiosis

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A rural community based case control study was conducted in Malawi, over a 23 month period, to identify determinants influencing human cryptosporidiosis in under fives. 96 home interviews were conducted in 24 communities (cases n=24; unmatched controls n=72). 61 risk factors were investigated by questionnaire, combined with quantitative data from drinking water and domesticated animal stool samples. Cryptosporidium oocysts were not detected in either sample type. Multivariate logistic regression of questionnaire data revealed an increased risk of cryptosporidiosis associated with ownership of pigs (OR7.2, 95%CI 1.9–27.5, p=0.004), presence of diarrhoea in the household (OR8.8, 95%CI 1.8–53.4, p=0.008), bathing in the river (OR76.7, 95%CI 1.1–23.8, p=0.037) and no education within the household (OR3.6, 95%CI 1.1–11.8, p=0.038). Bacteriological results indicating faecal contamination of both drinking water stored within the home, and the surface of guardians' hands were indicative of poor hygienic practices and potential sources of infection.

Introduction

Most studies assessing *Cryptosporidium* infections in the tropics, including Malawi, have focussed on the incidence of infection in hospitals and health centres. Few studies have investigated the sources of infection within a population, or suggested control measures to reduce the risk of disease in developing countries. There are currently 16 valid species of *Cryptosporidium* of which eight have been reported to infect humans.

Cryptosporidium spp. can be transmitted from person to person and zoonotically, both directly and indirectly, through the environment. Links to direct transmission have been determined (Elsser, *et al.*, 1986; Navarette *et al.*, 1991; Siwilia, *et al.*, 2007) but, mainly by statistical association of disease with exposure to risk factors (Chunge *et al.* 1992; Newman *et al.*, 1999; Pereira *et al.*, 2002). Unlike the situation in the UK and USA, where zoonotic and waterborne transmission are described frequently, no conclusive evidence for these transmission routes has been offered in developing countries however, the presence of *Cryptosporidium* oocysts in domesticated and wild animals, and drinking water sources indicates a zoonotic potential also in the tropics (Hunter *et al.* 2003; Kelly *et al.*, 1997; Mtambo, *et al.* 1997; Molbak *et al.*, 1994; Niyenzi *et al.*, 2002). Currently, little, molecular-based information is available describing species infecting human-, non-human hosts and those found in the environment in developing countries.

The World Health Organisation (WHO) Health Village Initiative and Water Safety Plans provide set systems by which communities can improve their health holistically, including a reduction in cryptosporidiosis, but an understanding of disease prevalence and sources is required to identify appropriate controls and interventions (Howard, *et al.*, 2002; WHO 2006). This study was designed to determine risk factors for human cryptosporidiosis in a rural Malawian setting using both qualitative and quantitative data collection methods with a view to reducing the risk of infection within that rural community.

Methodology

A community based case control study was conducted between January 2001 and December 2002 in a rural district in Southern Malawi (Chikwawa). Positive cases were identified through a hospital based study (Morse, *et al.* 2007), and cases were defined as children under the age of five, with diarrhoea which contained *Cryptosporidium* oocysts by microscopy. Twenty four cases were identified for the community based aspect of the study. Three controls per case (3:1) were identified from the same village as each case. Controls were under the age of five years and had no evidence of diarrhoea in the two weeks prior to the interview. Controls were chosen at random and were not matched for age or gender.

Data collection methods

All interviews and sample collections were carried out within one week of the diarrhoeic stool sample being submitted at the appropriate health clinic. Permission was obtained for conducting all interviews and sampling. Data were collected from all participants in the form of a qualitative questionnaire, environmental samples, and stool samples. All data collection tools were the same for both cases and controls.

Questionnaires

Questionnaires were translated into the local language and pre-tested. Questionnaires covered the areas of socio-economic status, housing standards, water use, personal hygiene practices, sanitation, food hygiene and animals contacts, and conducted with the primary child care giver (guardian).

Water samples

Water samples were procured from both the household drinking water container and the water source to determine the presence of coliforms, *E. coli* and *Cryptosporidium* spp. oocysts.

Testing for coliforms and *E. coli*

One sample was procured from each household drinking water storage container and three samples were procured from each water source. Water samples were collected in 100ml IDEXX Colilert® bottles, sealed at the point of sampling, then stored in cool boxes until analysed at the laboratory (University of Malawi – The Polytechnic). The presence/absence of coliforms and *E. coli* was determined using the IDEXX Colilert® substrate (IDEXX Laboratories, 2003).

Testing for *Cryptosporidium* oocysts

A one litre sample was procured from household drinking water storage containers and water sources. Samples were stored in sterile sample bags below 40C and returned to the testing laboratory (University of Malawi – The Polytechnic). Samples were stored below 40C for a maximum of 7 days prior to being concentrated to 2ml pellets (repeated centrifugation at 2500 x g for 10 min followed by aspiration of the supernatant). Pellets were resuspended in distilled water, and the final, resuspended, concentrate was stored in 2 ml microcentrifuge tubes, below 40C then forwarded to the Scottish Parasite Diagnostic Laboratory (SPDL), Glasgow, UK for further processing. Concentrates were resuspended and subjected to immunomagnetic separation (IMS; DYNABEADS™, Dynal Biotech Ltd, Warral, UK) according to the manufacturer's instructions. The IMS concentrate was deposited as two 50 µl aliquots onto two wells of a four well slide, then air dried. The presence/absence of oocysts was confirmed by immunofluorescence (IF) using a fluorescein isothiocyanate monoclonal antibody (FITC-C-mAb) kit (Cryptoglo, Waterborne Inc. New Orleans, USA), and the nuclear fluorogen 4'6-diamidino-2-phenyl indole (DAPI), according to Grimason et al. (1994). Nomarski differential interference contrast (DIC) microscopy was used to determine internal morphology (x1000 magnification).

Hand washing samples

Mothers/guardians were asked to wash their hands in a bag containing sterile water using small sponges which facilitated organism removal. Sponges were sterilised at 1210C for 15 min in the bags which also contained 500ml of tap water, and which remained sterile until used on site. 100 ml of handwash water was then decanted into an IDEXX Colilert® container. The presence/absence of coliforms and faecal coliforms was determined using the IDEXX Colilert® (IDEXX laboratories, 2003).

Animal stool samples

Samples of animal stools were collected from within the perimeter of the household. Once the species of animal excreting the stool was identified, a sample of stool was collected into a sterile container. All samples were subject to formal ether concentration (Allen and Ridley, 1970) and the concentrate deposited onto a microscope slide, air dried, then methanol fixed. Fixed samples were stained with modified Ziehl Neelsen (mZN; Casmore *et al.*, 1991) and examined for the presence of *Cryptosporidium* oocysts (x400 magnification).

Human stool samples

Cryptosporidium oocysts were detected in direct faecal smears by mZN and auramine phenol (AP) staining (Casmore, 1991). Subsamples of all stools were stored without preservative below 4°C and subjected

to further microscopic and molecular analyses at the SPDL (Morse *et al.*, 2007). Putative mZN and/or AP positive samples were confirmed by immunofluorescence as described above, at SPDL.

Data analysis

Data were entered and analysed using SPSS 13.0. For dichotomous data, the odds ratio and confidence intervals were estimated using the Mantel – Hasenzel χ^2 test. For continuous data, logistic regression of variables was carried out and were summarized by logistic regression odds ratios ($\exp(\beta)$) and 95% confidence intervals. A multivariate logistic regression model was devised to include variables which had a *p* value <0.25 in the initial univariate analysis and to compensate for possible confounding factors. Variables with a *p* value ≥ 0.05 were subsequently excluded from the model by stepwise regression analysis. Analysis was only performed on the presence / absence of *Cryptosporidium* oocysts in the stool, as sample numbers were too small to carry out full molecular analysis on the various *Cryptosporidium* species found (Morse *et al.*, 2007).

Results and discussion

During this 23 month study, a total of 96 home interviews were conducted in 24 villages in Chikwawa District (cases *n* = 24; controls *n* = 72). The mean age of participants was 14.3 months (S.D. 9.457) with 87% of participants being <24 months old. 45% of participants were female.

Data affecting individual risk factors were assessed and compared to national data where appropriate. A total of 61 risk factors were assessed for their effect on the presence/absence of cryptosporidiosis (defined by oocysts being present in diarrhoeic stools). Analysis conducted to identify determinants for cryptosporidiosis was limited by sample number. Six variables were discounted from analysis as they were consistent for both cases and controls. Thirty variables were included within the multivariate logistic regression based on significance. The *R*² values indicated that the model only accounted for 28 – 41% of cryptosporidiosis cases [0.28 (Cox and Snell) and 0.414 (Nagelkerke)]. This low variance indicates that, while this model can identify determinants which influence the acquisition of childhood cryptosporidiosis, we cannot infer that they are the sole risk factors. Some important limitations in our data collection included the lack of height and weight data to assess malnutrition levels, the lack of awareness of HIV status in children, and the lack testing for other concurrent infectious (viral, bacterial and parasitic) diseases.

Socio economic status

No education in the household was found to be an implicating factor in infection [OR = 3.6: CI(95%) 1.1 – 11.8, *p* = 0.04]. The total lack of education has not been reported as a determinant for cryptosporidiosis, previously, and this positive correlation is indicative of further risk factors which may be influenced by education, such as poor personal hygiene and poor child care. Occupation, house construction, the number of occupants in a house (including overcrowding), the numbers of siblings for a case were not significant for cryptosporidiosis in any model.

Drinking water

Sources

Drinking water is a well established route of *Cryptosporidium* transmission in developed countries and has been predominantly associated with supplies which are subject to insufficient treatment before consumption (Smith and Grimason, 2003). In sub-Saharan Africa, the potential for waterborne transmission was identified by Kelly, *et al.*, (1997), who demonstrated a significant association between increased cases of cryptosporidiosis and consumption of drinking water in affected areas of Zambia. Statistical associations between water consumption and cryptosporidiosis were also reported in the sub-Saharan region (Gascon, *et al.*, 2000). Five types of drinking water source, representing 28 separate supply points, were used by respondents in this study, namely boreholes (*n* = 17), unprotected shallow wells (*n* = 2), gravity tap systems (*n* = 6), protected well (*n*=1) and rivers (*n* = 2). When divided into protected and unprotected sources, all unprotected and 25% of protected water sources were contaminated with *E. coli*. These were predominantly associated with gravity fed systems, which collected water to a settlement tank from a catchment area and which was subsequently distributed to taps located within the surrounding communities. This water was not subjected to treatment. Although the presence of *E. coli* is not an indicator of the presence of *Cryptosporidium*, it does indicate that a water system is faecally contaminated, possibly following indiscriminate defaecation by human- and non-human sources. Oocysts can remain viable in human and animals stools for up to six months (Jenkins, *et al.*,

1999; Robertson, *et al.*, 1992), and when exposed to rainfall, can be released from faeces in high concentrations, being transported by runoff into drinking water systems (Davies, *et al.*, 2004; Schijven, *et al.*, 2004). Thus, if oocyst-contaminated faeces are present within the water catchment area, they can be transported into the water system from which the gravity fed system is derived. A seasonal increase in oocysts contaminating drinking water systems correlates with the seasonality of infection (Morse *et al.*, 2007).

The type of water source, and the microbiological quality of the drinking water at source did not have a significant effect on childhood cryptosporidiosis.

Drinking water in the home

All respondents collected water in metal buckets (ndowa) and stored it in locally produced clay pots. Microbiological examination of drinking water from clay pots demonstrated that, despite collection of drinking water from a 'clean' source, 76% of household water was faecally contaminated. Immersion of hands and utensils has been reported to be an associated risk factor in the contamination of drinking water (Swerdlow *et al.*, 1997; Trevett, *et al.*, 2005a; 2005b). In our study, three out of four guardians' hands were contaminated with *E. coli* at the time of interview. This high level of faecal contamination enhances the likelihood for water contamination during collection, storage and retrieval of drinking water, and requires further investigation.

Nine of ten (90.6%) children consumed drinking water stored within the household, three quarters of (74.7%) whom drank *E. coli* contaminated drinking water. However, a child who drank household water did not have a significant association between presence/absence of cryptosporidiosis [OR = 0.4: CI(95%) 0.01 – 1.5, $p = 0.17$], although drinking water is a potential vehicle for transmitting diarrhoeal diseases, including cryptosporidiosis.

Appropriate treatments can reduce the contamination of household drinking water by pathogens. With respect to the risk of contracting cryptosporidiosis from household water, our questionnaire concentrated on boiling as an effective treatment for rendering oocysts non infectious. Although a small percentage (14.6%) of respondents indicated that they boiled drinking water prior to consumption, little improvement was detected in the microbiological quality of their household drinking water (40% *E. coli* positive). This may be as a result of post treatment contamination, inadequate boiling or providing 'expected answers' to the interviewer. Environmentally and economically friendly, alternative, treatment systems exist for disinfecting *Cryptosporidium* and other diarrhoeal agents. Mendez – Hermida, *et al.*, (2005) demonstrated that, under laboratory conditions, solar disinfection can render *Cryptosporidium* oocysts non infective after 12 h of exposure.

The control of water safety within the rural community lies with the health surveillance assistants (government employed) and the water point committees (volunteers). Their training must include details of Water Safety Plans in the future to provide them with the necessary skills to identify and assess hazards within their communities and subsequently control them at source, collection and household level. Such capacity building at community level could help to reduce the reliance on non governmental organisations and district water offices in the control of safe drinking water (WHO, 2006).

Cryptosporidium oocysts were not isolated from either source or household water samples. The sampling method used was simplistic and limited because of the equipment and facilities available within Malawi. Small volume samples containing fewer oocysts than large volume reduces oocyst isolation, while long term storage of sample concentrates (maximum 2 years) can influence oocyst structure, thereby reducing the efficacy of the IMS and IF systems we used. Further sampling of water for oocysts should include the collection of larger volumes where possible, and the use of an additional or alternative concentration methods such as membrane filtration or CaCO₃ flocculation (Vesey *et al.*, 1991) to increase oocyst recovery.

Sanitation

More than half of all respondents (52.2%) did not have pit latrines. No significant association between pit latrine ownership, pit latrine structure, the disposal of child excreta and the presence of *Cryptosporidium* oocysts in cases was found in this study. Previous studies identified poor sanitation and lack of latrines as determinants for *Cryptosporidium* transmission in sub-Saharan Africa (Gascon, *et al.*, 2000; Nizeyi, *et al.* 2002). Traore, *et al.*, (1994) demonstrated that the risk of children contracting diarrhoeal illnesses was 35% higher in individuals living in households where human faeces were observed on the ground, while van Derslice *et al.* (1994) found that reducing environmental contamination with faeces reduced the risk of diarrhoeal illness. As such, the indiscriminate disposal of faeces cannot be discounted as a potential route for the direct or indirect transmission of *Cryptosporidium* spp. oocysts.

Studies on the disposal and handling of excreta in Malawi identified lack of funds and lack of technical expertise as major factors in the absence of latrines (Grimason, *et al.*, 2000). As such, household members, particularly in rural areas, may chose to defaecate in open areas, or construct poor quality latrines that may be abandoned due to collapse or malodours. As no sanitation policy exists presently within Malawi, there is little guidance for households on the benefits of latrines, their placement or construction, which leads to little encouragement for their use.

The presence of filth flies and cockroaches in poorly maintained latrines also offer a further transmission route for diarrhoeal disease agents including *Cryptosporidium* spp. Studies have demonstrated that adult and larval stages of filth flies which fed on oocyst contaminated faeces can transmit *Cryptosporidium* oocysts to distant surfaces in their faeces and following direct contact with their contaminated outer surfaces (Gracyk, *et al.*, 1999; Getachew, *et al.*, 2007). The ubiquitous presence of flies in the latrine, home and surrounding area must be considered as a potential route of infection for cryptosporidiosis, particularly in young children, where the infectious dose required for disease manifestation may be low (DuPont, *et al.*, 1995).

Household hygiene

We assessed various aspects of household hygiene, including personal hygiene, food hygiene, and management of diarrhoea.

Personal hygiene

Respondents were asked open questions with regard to hand washing practices to reduce the likelihood of giving 'expected answers'. The majority (84.4%) of guardians indicated that they washed their hands after using the toilet and before preparing/eating food (85.4%), but no guardian stated that hands were washed after handling their child's faeces. Hand washing prior to eating is a consistently observed custom in Malawi (personal observation) however, washing hands after visiting the toilet is perhaps not as consistent. Mariun'Ebo, *et al.*, (1997) reported that mothers over reported hand washing practices, and a similar effect cannot be ruled out here. Although the majority of respondents were aware that hands should be washed after defaecating, the practical limitations of hand washing reduce the probability of performing the practice. Here, systems using locally available products should be encouraged, yet, during the period of this study, the majority of households used traditional methods of hand immersion in basins used by several persons, and pouring of water over hands, which requires two persons for hand washing, while only one innovative system was seen. Poor personal hygiene practices are risk factors for contracting cryptosporidiosis in developing countries, and are associated with institutional settings such as hospitals and day care centres (Navarette, *et al.*, 1991). Improved maternal hand washing reduces the incidence of diarrhoea in children (Han and Hlaing 1989).

The presence of soap in a household is significantly associated with the reduction of diarrhoea in household members (Peterson *et al.*, 1998). In our questionnaire, the use of soap for hand washing did not have a significant effect on the absence of *Cryptosporidium* in this study. Only 27.1% of guardians possessed soap at the time of interview and soap usage varied widely between cases and controls [OR = 0.3: CI (95%) 0.1 – 1.1, $p = 0.06$]. Low soap usage may be a reflection of the low socioeconomic status of families within the rural communities of Chikwawa. Apart from hand washing, soap will be used for household duties including cleaning food utensils, washing clothes and bathing, and as such, using soap for hand washing may be low priority. Effective, low cost alternatives to soap are available. Ash can improve the efficacy of water to remove dirt and microorganisms from skin (Hoque *et al.* 1991). The promotion of ash as a hand cleaning agent requires to be addressed within communities at health education sessions in order to improve the efficacy of hand washing.

Three-quarters (75%) of participants were found to have faecal contamination on their hands, however this was not found to be a determining factor for cryptosporidiosis. Nevertheless, this level of contamination is indicative of poor hand washing practices, and may indicate an incorrect reflection of practices identified by guardians in the questionnaire. Hand cleanliness was not associated with the presence of soap in the household ($\chi^2 = 1.1$, $df = 1$, $p = 0.29$), or the method of hand drying [shaking: ($\chi^2 = 0.06$, $df = 1$, $p = 0.81$); cloth: ($\chi^2 = 0.06$, $df = 1$, $p = 0.81$)]. Faecally contaminated hands may enhance person to person transmission of diarrhoeal diseases, particularly to susceptible, young children, and, in our study, the presence of diarrhoea in other household members was a significant determinant for the presence of cryptosporidiosis in children less than five years [OR=8.8: CI (95%) 1.8 – 53.4, $p=0.008$], indicating the importance of person to person transmission for cryptosporidiosis. In one household, both the case and a sibling excreted *C. hominis* oocysts. Studies conducted in other developing countries have shown a similar association between diarrhoea

/ cryptosporidiosis in the home and transmission to other members of the household (Newman, *et al.*, 1994; Pereira, *et al.*, 2002; Rahman, *et al.*, 1985).

Bathing of children was undertaken in a bucket or basin in the home (89.6%), in a bathing shelter at the home (2.1%) or in a nearby river (8.3%). Following multivariate analysis of risk factors for cryptosporidiosis, only bathing in a river was found to be a determining factor for cryptosporidiosis (OR= 6.7: CI (95%) 1.1 – 23.8, $p = 0.04$). The multiple use of open water systems, such as rivers, for bathing, washing clothes, retrieving drinking water, and as a source of drinking water for domesticated (and feral) animals, makes them ideal sources for the transmission of diarrhoeal diseases.

Grimason, *et al.*, (2000) pointed to the urban Malawian phenomenon whereby one third of respondents believed that stools from babies were 'less harmful' than those of adults and therefore did not require 'proper' disposal. This belief may also have influenced the fact that no guardians indicated that they washed their hands after handling excreta from children. Appropriate disposal of stools from children was found to significantly reduce household diarrhoea in Burkina Faso (Curtis, *et al.*, 1997). The method of disposal of child faeces was not found to be a significant risk factor in the presence/absence of cryptosporidiosis.

Food hygiene

Poor food hygiene has been long associated with the transmission of diarrhoeal diseases. Foodborne cryptosporidiosis has been associated with street vended foods, fruit, vegetables and unpasteurised milk in tropical countries (Elsser, *et al.*, 1986; Quiroz, *et al.*, 2000; Sterling, *et al.*, 1986; Monge, *et al.*, 1996; Ortega, *et al.*, 1997). The location of fruit and vegetable vendors, the use of fertilisers, washing of fruit and vegetables before consumption, the specific area for food preparation in the home were not significant risk factors for cryptosporidiosis. However, the presence of oocysts on locally produced fruit, vegetables and meat cannot be discounted: carcasses are exposed to faecal matter and filth flies during slaughter, faeces can contaminate water, soils, sewage, while poor food handling / personal hygiene also increases the risk of foodborne contamination. 80.2% of children in our study were breastfed (cases = 75%; controls = 82%), but no significant difference was found between the incidence of cryptosporidiosis and the practice of breastfeeding [OR = 0.7: CI (95%) 0.2 – 2.0, $p = 0.5$]. Childhood *Cryptosporidium* infection rates increase up to two years of age, after which it is thought they achieve active immunity from repeated exposure to infection. Breastfeeding children up to six months old can reduce the risk of cryptosporidiosis in this age group (Simwa *et al.*, 1989; Zu *et al.*, 1994).

Domesticated animals

Livestock, particularly cattle and sheep, are a reservoir for the zoonotic transmission of *C. parvum*, (Konkle, *et al.*, 1997; Smith, *et al.*, 2004). No *Cryptosporidium* oocysts were detected in 195 samples of animal stools using mZN. *C. parvum* oocysts were isolated from cattle in Chikwawa District in 2002 indicating both the presence of this zoonotic species and the potential for disease transmission within the rural population of this District (Banda unpublished data). Siwilia, *et al.*, (2007) also reported the zoonotic transmission of *Cryptosporidium parvum* between dairy farm workers and cattle in Zambia. *Cryptosporidium* oocysts have been isolated from pigs in other countries (Enemark, *et al.*, 2003; Nunez, *et al.*, 2003).

The ownership of cattle, pigs and cats was higher in cases than in controls (Cow: Cases $n = 20.8\%$, Control $n = 6.9\%$; Pig: Cases $n = 41.7\%$, Controls $n = 9.7\%$; Cats: Cases $n = 41.7\%$, Controls $n = 15.3\%$). On the basis of the number of animals observed around each home and the number of stools observed, exposure to animals was similar in cases and controls. Ownership of pigs was found to be a significant determinant of cryptosporidiosis in cases [OR = 7.2: CI (95%) 1.9 – 27.5, $p = 0.004$].

Statistical associations between cryptosporidiosis and animal contact have been reported previously in the tropics. Cattle, pig, dog and cat and pig ownership was reported to be a significant risk factor for the presence of cryptosporidiosis in Guinea Bissau (Cartensen, *et al.*, 1987; Chungue, *et al.*, 1992; Molbak, *et al.*, 1994; Nyezi, *et al.*, 2002).

Conclusion

This study builds an effective picture of faecal-oral disease transmission in rural Malawi. The use of qualitative, quantitative and observational data has allowed the demonstration of critical disease transmission routes, not only for *Cryptosporidium*, but also, for other diarrhoeal disease agents. The importance of water hygiene, particularly after collection, animal control, sanitation and hygiene education are highlighted as key areas which require attention at community level if disease transmission is to be reduced in the under fives. Implementation of programmes such as the WHO Healthy Village concept in conjunction with Water

Safety Plans to build capacity and provide a holistic approach to health improvement at rural level in Malawi are essential in diarrhoeal disease reduction.

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