Unexpected results from large-scale cryptosporidiosis screening study in calves in Tanzania

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A study was undertaken to investigate *Cryptosporidium* infection in crossbreed dairy calves in two districts in Tanzania. A total of 943 fecal samples from 601 dairy calves were included in the study, with calves from both smallholder dairy farms and from large-scale and medium-scale dairy farms. The modified Ziehl–Neelsen (mZN) technique was used to examine 710 samples, and 13 of these were considered to be positive for *Cryptosporidium*. These 13 samples considered positive by mZN, along with the remaining 233 samples, were analysed by immunofluorescent antibody test (IFAT). Of these 246 samples examined by IFAT, 15 samples, 10 of which were considered positive by mZN, were also examined by the auramine phenol technique, and 5 samples, all of which were considered positive by mZN, were analysed by PCR. The results from the IFAT, auramine phenol and PCR analyses demonstrated that none of the samples contained *Cryptosporidium* oocysts, indicating that, cryptosporidiosis is currently not a problem in dairy calves in these regions of Tanzania. These unexpected results are discussed with respect to other reports on cryptosporidiosis in calves that suggest that this parasite is a serious calf disease globally, and particularly in relation to studies from Tanzania. We suggest that results from studies of cattle in Tanzania, in which mZN has been used as the sole analytical method, should be treated with caution.

INTRODUCTION

Cryptosporidium is a parasitic protist that causes intestinal infections and clinical disease in both humans and animals worldwide. Some species of *Cryptosporidium*, particularly *C. parvum*, are well known as zoonotic pathogens, being especially associated with human and cattle infections (mainly in young calves), while other species of *Cryptosporidium* are more or less host

specific. For example, *Cryptosporidium hominis* is particularly associated with human infections, and *C. bovis* and *C. andersoni* particularly associated with infections in cattle (Xiao, 2010). Severe diarrhea occurs in young and immunocompromised animals and humans (Gatei *et al.*, 2003; Ramirez *et al.*, 2004). However, self-limiting or subclinical disease also occurs, predominantly in adults and immunocompetent animals and humans (Ramirez *et al.*, 2004).

Cryptosporidium infection can play an important role in animal production, as it can have a negative influence on growth and impair feed conversion (Esteban and Anderson, 1995; de Graaf *et al.*, 1999).

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Neonatal infections may result in significant economic losses due to their negative effects on the growth rate.

Previous studies from East Africa (Kenya and Tanzania) have indicated prevalences of *Cryptosporidium* infections in cattle ranging between 0.5 and 35% (Table), but there has been little research conducted on the species detected. This is important, not just from the veterinary point of view, but also because of the zoonotic potential. The aim of this study was to determine the prevalence and species of *Cryptosporidium* infection in crossbreed dairy calves in Njombe and Mvomero districts, Tanzania. The intention was to use the data to enable assessment of the risk of this disease to cattle and humans in these regions, and determine whether this common infection of calves contributes to the compromised health and welfare of calves as diarrheal disease was

ranked the second most common disease condition in calves in Njombe and Mvomero (Chang'a *et al.*, 2010).

MATERIALS AND METHODS

Study Area

The study was carried out in two districts of Tanzania, Mvomero in southeastern Tanzania (latitude: 5°35'S; longitude: 37°58'E) and Njombe in the southern highlands (latitude: 9°19'S; longitude: 34°46'E). Further details of the study sites have been published previously (Chang'a *et al.*, 2010).

Sample Collection

A total of 943 fecal samples were collected from 601 calves (3 days old to 57 weeks of age), of which 731 samples were from 389

TABLE. Studies on *Cryptosporidium* infection in cattle in Tanzania and Kenya, including prevalence and detection method used

Sample details	Percentage prevalence (N)	Number of samples examined	Detection method	Study location and reference
Calves from smallholder dairy herds and traditional herds	35	117	mZN ^a	Tanzania; Swai and Schoonmann, 2010
Dairy cattle from two regions	19.7	1126	mZN	Tanzania; Swai <i>et al.</i> , 2007
Cattle (age not specified; kept under one of two different management systems)	0.5	942	mZN	Tanzania; Kusiluka <i>et al.</i> , 2005
Cattle (calves, weaners and adults)	Overall prevalence: 5.3 Calves: 16.3 Weaners: 3.3 Adults: 1.4	Total: 486 Calves: 116 Weaners: 91 Adults: 279	mZN, positives confirmed with IFAT ^b	Tanzania; Mtambo <i>et al.</i> , 1997
Calves from urban and peri-urban households	23.8	143	mZN, but only 2 positive by PCR ^c out of 34 positive by mZN	Kenya; Szonyi <i>et al.</i> , 2008
Dairy cattle and calves (pooled samples)	18	285	mZN	Kenya; Kang'ethe <i>et al.</i> , 2007

^amZN: modified Ziehl-Neelsen.

^bIFAT: immunofluorescent antibody test.

^cPCR: polymerase chain reaction.

smallholder dairy calves and 212 samples from calves belonging to either a large-scale dairy farm, or one of two medium-scale dairy farms. All calves were crossbreeds, being Ayrshire, Friesian or Jersey crossed with East African Zebu. Samples were examined for *Cryptosporidium* by use of between 1 and 3 techniques per sample, as described in the sections below. For 445 calves, a single sample was examined once (cross-sectional prevalence), while 156 calves were included in a longitudinal study, in which up to four samples were examined, with samples collected every 2 months for 1 year, giving a total of 498 samples.

Fecal samples were collected per rectum using disposable gloves and transported in a cool box to the laboratory at the Sokoine University of Agriculture (SUA), Morogoro. Approximately 6.2% of the samples were classified as diarrheic.

Sample Analysis by Modified Ziehl-Neelsen Staining

Modified Ziehl-Neelsen (mZN) staining, as described by Henriksen and Pohlenz (1981), was used at SUA to identify samples containing objects resembling *Cryptosporidium* spp. oocysts. Fecal smears were prepared on a microscope slide, air-dried and fixed with methanol for 5 minutes. Fixed smears were stained with strong carbol fuchsin for 3–5 minutes and washed with tap water. Smears were decolorized using acid alcohol, then counterstained with 0.5% malachite green solution for 1 minute. The smears were air-dried and examined using bright field microscopy at $\times 400$ magnification. A total of 710 samples were initially examined by mZN.

Sample Analysis by Immunofluorescent Antibody Test, Auramine Phenol Staining and PCR

The remaining 233 samples (not initially studied by mZN), along with 13 samples identified as containing putative *Cryptosporidium* oocysts by mZN, were analysed using immunofluorescent antibody test (IFAT)

(A100FLR from Waterborne Inc., New Orleans, LA, USA), with additional staining with a nuclear fluorochrome (4',6-diamidino-2-phenyl indole; DAPI). Examination of stained samples was by fluorescence microscopy [blue (FITC) filter for the immunofluorescence and UV filter for DAPI] at $\times 200$ magnification for initial screening and $\times 400$ magnification for closer examination. Samples analysed by IFAT were stained and examined at the parasitology lab at the Norwegian School of Veterinary Science in Oslo, Norway.

Of these samples, 15 were also examined by the auramine phenol technique (10 originally scored as positive by mZN, and 5 not previously examined by mZN), also at the parasitology lab at the Norwegian School of Veterinary Science in Oslo, Norway. For sample analysis by this method, fixed fecal smears were stained in phenol auramine for 10 minutes, rinsed with tap water, decolorized in 3% acid alcohol for 5 minutes and counterstained with 0.1% potassium permanganate for 30 seconds. Examination was by fluorescence microscopy (blue filter) at $\times 200$ magnification for initial screening and $\times 400$ magnification for closer examination.

For investigation by PCR, putative oocyst isolation was first conducted from five samples, selected from those scored as positive by mZN (but negative by IFAT), and with sufficient material for further testing. A modified immunomagnetic separation (IMS) procedure was used (GC-Combo; Dynal Invitrogen, Oslo, Norway) as previously described (Robertson *et al.*, 2006), in which a 1 ml suspension of fecal material was mixed (end-over-end rotation) for 1 hour with 100 μ l of each buffer provided in the kit and 15 μ l beads coated with anti-*Cryptosporidium* oocyst antibody. DNA was then extracted from the concentrate (after separation from the beads by vortexing in acid) using QIAmp DNA mini-kit (QIAGEN GmbH, Hilden, Germany) and following the manufacturer's protocol, but preceded by a 1 hour incubation at 100°C. PCR was conducted on the isolated DNA and directed towards a fragment, approximately 800 bp, of the SSU rRNA

gene of *Cryptosporidium*, using previously published primers and with only minor modifications to the published protocol (Xiao *et al.*, 1999). The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide. Two positive controls were included from the IMS stage of the study. One positive control was a fecal sample from a natural infection of a Norwegian calf with *C. parvum*, and the other was a negative fecal sample (by IFAT and mZN) from a Tanzanian calf from this study that was seeded with approximately 500 *Cryptosporidium* oocysts per ml faecal suspension. A negative control (a negative fecal sample from a Norwegian calf) was also included from this stage.

RESULTS

Of the 710 samples initially examined by mZN, 13 (1.4%) were suspected to contain *Cryptosporidium* oocysts. Of these 13 suspect-positive samples, 4 were diarrheic. However, on re-examination by IFAT none of these samples were found to contain *Cryptosporidium* oocysts. Similarly, all of the 233 samples analysed first by IFAT were found to be negative for *Cryptosporidium*. Likewise, only negative results were obtained by auramine phenol staining and also by PCR. Positive control samples for IFAT, auramine phenol and PCR gave positive results.

DISCUSSION

Although a number of calves were involved in this study, and cross-sectional and longitudinal approaches were used, with samples collected during both rainy and dry seasons, our results indicate that *Cryptosporidium* infection in these crossbreed dairy calves was below the limit of detection, and that cryptosporidiosis is therefore currently not an important disease in the calf populations in the areas of Tanzania involved in this study.

Initially, these results seemed very surprising, as a substantial number of studies have shown *Cryptosporidium* spp. infection to be highly prevalent among calves in different areas of the world (Sturdee *et al.*, 2003; Santin *et al.*, 2004; Hamnes *et al.*, 2006), including in various studies from Kenya and Tanzania (Table), and *C. parvum* and *C. andersoni*, in particular, are generally considered common parasites of cattle worldwide (Olson *et al.*, 2004; O'Handley and Olson, 2006), with *C. parvum* infections especially common in calves.

However, closer consideration of the situation suggest that perhaps the results described from this study are not so surprising, and that, indeed, cryptosporidiosis in cattle may currently not be a particularly important problem for calves in some areas of Tanzania. Various factors are important in this assessment and these must be considered carefully as this interpretation contradicts the general consensus of opinion regarding the importance of cryptosporidiosis in cattle, particularly calves.

Although cryptosporidiosis is important in the human population of East Africa, particularly in immunocompromised individuals, in studies in which the species of *Cryptosporidium* has been investigated, the vast majority of infections in people in sub-Saharan Africa appear to be with *C. hominis* (Mor and Tzipori, 2008; Chako *et al.*, 2010). This information, in combination with an apparent lack of difference in prevalence of *Cryptosporidium* infections between urban and rural populations in sub-Saharan Africa (Mor and Tzipori, 2008), suggests that human infection is largely anthropogenic, and, thus, that zoonotic infections in cattle are perhaps rather limited. It should, however, be noted that in Zambia, where cryptosporidiosis is considered to be a major contributor to childhood mortality and morbidity, zoonotic transmission from cattle is considered to occur (Kelly, 2011).

The reasons that endemic *Cryptosporidium* infections may have failed to establish in cattle in some areas of Tanzania, would be

almost certainly multi-factorial, and probably include demographic and environmental factors, as well as factors related to cattle management. Herd prevalence of *Cryptosporidium* tends to increase with increasing herd size (Mohammed *et al.*, 1999; Hamnes *et al.*, 2006), but many of the individual farmers in this study owned just a couple of animals and thus, potential sources of infection are relatively low. Low herd size and lack of grazing also reduce the potential for farm to farm transmission, and can result in fade out of disease (Anderson and May, 1992). Additionally, oocyst survival in some areas of Tanzania may be relatively brief. Oocyst survival is known to be optimal under cool, damp conditions, and the high ambient temperature, significant solar radiation and prolonged periods of drought associated with many regions of Africa, may result in rapid oocyst inactivation. Indeed, high temperature and increased solar radiation has been demonstrated to enhance *Cryptosporidium* oocyst inactivation in the environment (Fujino *et al.*, 2002). Intriguingly, two recent studies from West Africa (Nigeria) have demonstrated the occurrence of *Cryptosporidium* infection in Nigerian cattle herds, but only *C. bovis*, *C. ryanae* and *C. andersoni* were found, and *C. parvum* was not detected (Ayinmode *et al.*, 2010; Maikai *et al.*, 2011). As almost all environmental survival studies have been conducted on oocysts of *C. parvum*, it is possible that oocysts from other species of *Cryptosporidium* are better able to tolerate dry, warm conditions.

Another factor that may be of importance is host species. In this study, all the calves were crossbreeds, being Ayrshire, Friesian, or Jersey crossed with East African Zebu. Thus, these cattle are crossbreeds of *Bos primigenius taurus* and *Bos p. indicus*, while the majority of other studies on *Cryptosporidium* in cattle tend to involve different breeds of *B. p. taurus*. It is possible that pure *B. p. taurus* cattle may be more susceptible to *Cryptosporidium* infection. Indeed, it has previously been suggested that some *B. p. indicus* breeds

may be more resistant, although the data are scanty (Maikai *et al.*, 2011). However, other studies have reported *Cryptosporidium* infection from both *B. p. indicus* and crossbreed cattle (Swai and Schoonmann, 2010).

Nevertheless, although these factors suggest that *Cryptosporidium* infection may not have established in some of the cattle populations in sub-Saharan Africa, the data from previous surveys in this region (Table), and that have indicated prevalences ranging from 0.5% up to 35% (median prevalence of six published studies: 19%), must also be considered.

In the present study, in which a large number of samples were to be screened, a rapid, cheap screening method was initially chosen (mZN), despite being known to have a relatively low detection sensitivity (Arrowood and Sterling 1989; Quilez *et al.*, 1996). However, the number of false positives that were detected was unexpectedly high, and we suggest that false-positives from mZN may partly explain the rather higher prevalences published in other studies from Tanzania and other countries in East Africa. Our studies suggest that other objects occur in bovine feces in Tanzania that are the same size as *Cryptosporidium* oocysts and which absorb acid-fast stains. Indeed, Arrowood and Sterling (1989) also reported problems with false-positive results using acid-fast staining techniques, and some fungal spores have been found to be acid-fast (Casemore, 1991; Sunnotel *et al.*, 2006). A study that compared three methods of detecting *Cryptosporidium* oocysts in cattle feces (mZN, auramine phenol staining and a commercial enzyme immunoassay kit), found that although mZN had a comparable or better sensitivity than the other methods, the specificity was lower (Brook *et al.*, 2008), and the main advantages of this method, according to these authors, were its price and the ease of keeping the slides as a permanent record.

In a study from Kenya on *Cryptosporidium* infection in cattle kept under urban and peri-urban conditions, out of 143 samples analysed by mZN, 34 (24%) were considered to

be positive, but of these, only 2 were positive by PCR (Szonyi *et al.*, 2008), and the authors themselves suggest that the non-specific staining could explain this result; these results are in accordance with our findings. It is also interesting to note, that of the two samples positive by PCR, both were *C. ubiquitum* (deer-like genotype), which although infectious to a wide range of potential hosts, including cattle and people, is more frequently associated with infection in sheep and cervids (Fayer *et al.*, 2010).

Thus, it appears that the sensitivity and specificity of mZN in detecting *Cryptosporidium* oocysts may vary, presumably depending on a range of matrix variables, including the occurrence of acid-fast oocyst-sized objects or organisms within the fecal samples. As almost all the studies on *Cryptosporidium* in cattle in Tanzania have relied solely on mZN for detection (Table), our results, taken in combination with the factors outlined above, suggest that some of these previously published data should be treated with caution, although we fully accept that regional and seasonal variation probably also contribute to the discrepancies in results. Cattle breed may also be important, with some breeds being more susceptible to infection than others.

In conclusion, this study demonstrates that cryptosporidiosis is currently not a problem in dairy calves in some regions of Tanzania. We realize that this information contradicts currently accepted norms regarding *Cryptosporidium* in calves, and suggest that the epidemiology of *Cryptosporidium* infection in cattle, humans and other animals in East Africa in general, and in Tanzania in particular, is highly worthy of closer investigation, using the most sensitive detection technologies available, and with molecular analysis of positive samples.

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