



***Cryptosporidium* infection in non-human hosts in Malawi**

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ABSTRACT

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Of 1 346 faecal samples from the Chikwawa and Thyolo districts of Malawi, analysed for the presence of *Cryptosporidium* oocysts between October 2001 and May 2003, 61.3 % were from cattle (29.8 % of these were from calves <6 months old). *Cryptosporidium* oocysts were detected during all three seasons studied in Chikwawa and Thyolo. In Chikwawa, 13.6 % of adult cattle and 11.7 % of calves were infected, compared to 28.9 % of adult cattle and 36.7 % of calves in Thyolo. Dependent on season, between 7.8 % and 37.7 % (Chikwawa) and 16.7 % and 39.3 % (Thyolo) of cattle samples contained oocysts. In Chikwawa, the highest percentage of infections occurred in the cool season, whereas in Thyolo, the highest percentage of infections occurred in the dry season. Faecal samples from goats [$n = 225$], pigs [$n = 92$], sheep [$n = 6$], rabbits, guinea pigs, chickens, ducks, turkeys, doves and guinea fowls were also analysed. Up to 5.6 % of goat samples contained oocysts in Chikwawa, compared to between 16.7 % and 39.3 % in Thyolo. Again, in Chikwawa, the highest percentage of infections occurred in the cool season and the lowest in the rainy season, whereas, in Thyolo, the highest percentage of infections occurred in the dry season and the lowest in the cool season. In pigs, more infections were detected in the dry season in Chikwawa, but infections in the cool season were similar (17.7 %), whereas in Thyolo, infections occurred in all three seasons (17.9 % in the rainy season, 25 % in the cool season and 60 % in the dry season). Of ten diarrhoeic, oocyst positive cattle faecal samples collected from Chikwawa and subjected to PCR-RFLP, four oocyst positive samples (two from heifers, one from a cow and one unknown) were amplified at an 18S rRNA and *Cryptosporidium* oocyst wall protein (COWP) loci. RFLP of the 18S rRNA locus indicated that *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Cryptosporidium bovis* and / or *Cryptosporidium ryanae* DNA, or a mixture of them was present. *Cryptosporidium parvum* DNA was identified in one sample that amplified at the COWP locus, indicating the presence of the major zoonotic *Cryptosporidium* species in Malawi.

Keywords: Cattle, *Cryptosporidium parvum*, livestock, Malawi, oocysts, season

INTRODUCTION

The apicomplexan parasite, *Cryptosporidium*, is responsible for many outbreaks of human disease

affecting both immunocompetent and immunocompromised individuals (Cacciò, Thompson, McLauchlin & Smith 2005). Cryptosporidiosis is also of veterinary importance since farm animals can be infected with various *Cryptosporidium* species that cause morbidity, with subsequent economic consequences (Smith 2008). *Cryptosporidium* has a large range of host species and infected hosts can shed large numbers of viable, environmentally robust oocysts that contaminate wastewater, watersheds, surface and ground waters and foods (Fayer 2008; Smith, Cacciò, Cook, Nichols & Tait 2007). In humans inhabiting many regions of the world, *Cryptosporidium*

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constitutes part of the complex group of parasitic, bacterial and viral diseases that impair the ability to achieve full potential and impair development and socio-economic improvements. All diseases included in the World Health Organisation (WHO) Neglected Diseases Initiative have a common link with poverty and, as the current view is to take a comprehensive approach to all these diseases, *Cryptosporidium* was included in the 'Neglected Diseases Initiative' in 2005 (Savioli, Smith & Thompson 2006).

The epidemiology of *Cryptosporidium* is complex with many hosts harbouring more than one species that can cross host species barriers, and having at least one host-adapted species, which are less likely to cross host barriers (Fayer 2008; Smith 2008). Of the 21 recognised *Cryptosporidium* species, humans can be infected with *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium muris*, *Cryptosporidium canis*, *Cryptosporidium suis* and the *Cryptosporidium* cervine, monkey, skunk and rabbit genotypes (Caccio *et al.* 2005; Nichols, Chalmers, Sopwith, Regan, Hunter, Grenfell, Harrison & Lane 2006), but two species, *C. parvum* and *C. hominis*, cause the majority of human disease (Caccio *et al.* 2005). In livestock, *C. parvum*, *Cryptosporidium andersoni*, *Cryptosporidium baileyi* and *C. meleagridis* cause disease (Smith 2008). *Cryptosporidium parvum* cryptosporidiosis is a cause of scours in young, unweaned livestock, and is the species recognized for having the greatest zoonotic potential.

Transmission of *Cryptosporidium* occurs following the ingestion of the environmentally robust transmissive stage, the oocyst. Transmission can be direct or indirect. Direct routes include person to person, animal to animal, and animal to person (zoonotic) and indirect routes include contaminated faeces and soil, surface and ground water, wastewater, feed, herbage, vectors and equipment (Cacciò *et al.* 2005; Smith, Cacciò, Tait, McLauchlin & Thompson 2006; Smith *et al.* 2007; Xiao & Feng 2008).

High mortality due to cryptosporidiosis has been reported in cattle mainly among meat breeds (Sanford & Josephson 1982; Peeters, Villacorta, Vanopdenbosch, Vanderghenst, Naciri, Ares-Mazas & Yvone 1992). *Cryptosporidium andersoni* infection in cattle is known to affect weight gain (Anderson 1987) and milk production (Esteban & Anderson 1995). Cryptosporidiosis in neonatal small ruminants such as goats and sheep can result in weight loss and retarded growth during the first few weeks of their life (Naciri, Yvone & Leieux 1984) and mortality has been reported in goat kids (Vieira, Silva, Tolentino, Lima

& Silva 1997) and adult goats (Johnson, Muirhead, Windsor, King, Al-Busaidy & Cornelius 1999).

Oocyst occurrence surveys identify the cosmopolitan nature of the parasite, occurring in numerous developed and developing countries (Fayer 2008; Smith & Grimason 2003; Gold & Smith 2002). In east Africa, studies performed in Kenya (e.g. Estambale, Bwibo, Kang'ethe & Chitayi 1989; Mwachari, Batchelor, Paul, Waiyaki & Gilks 2003; Simwa, Chunge, Kinoti, Karumba, Wamola & Kabiru 1989; Joyce, McGuigan, Elmore-Meegan & Conroy 1996; Peng, Matos, Gatei, Das, Stantian-Pavlinic, Bern, Sulaiman, Glaberman, Lal & Xiao 2001; Gatei, Greensill, Ashford, Cuevas, Parry, Cunliffe, Beeching & Hart 2003; Gatei, Wamae, Mbae, Waruru, Mulinge, Tabitha, Gatika, Kamwati, Revathi & Hart 2006), Uganda (Tumwine, Kekitiinwa, Bakeera-Kitaka, Ndeezi, Downing, Feng, Akiyoshi & Tzipori 2005) and Malawi (Morse, Nichols, Grimason, Campbell, Tembo & Smith 2007) have revealed infections in paediatric and adult immunocompetent and immunocompromised persons, but there are fewer studies investigating *Cryptosporidium* occurrence in non-human hosts (e.g. Mtambo, Sebatwale, Kamburaje, Muhairwa, Maeda, Kusiluka & Kazwala 1997; Nizeyi, Mwebbe, Nanteza, Cranfield, Kalema & Graczyk 1999; Graczyk, DaSilva, Cranfield, Nizeyi, Kalema & Pieniazek 2001). In the Chikwawa district of Malawi, an occurrence study in under-5-year-old children ($n = 848$) revealed that 5.9% of children had cryptosporidiosis, 10 (1.2%) of whom were infected with *C. parvum* (Morse *et al.* 2007).

At present, there is no information available regarding the prevalence of *Cryptosporidium* spp. in non-human hosts in Malawi. Owing to the importance of this parasite, and the potential for zoonotic transmission, a study was carried out in the Chikwawa and Thyolo Districts of Malawi with the main objective of determining the prevalence of *Cryptosporidium* spp. infection, primarily in cattle.

MATERIALS AND METHODS

Study areas

The study was carried out in Chikwawa and Thyolo Districts of the southern region of Malawi from October 2001 to May 2003. Chikwawa is ~50 km from the commercial capital of Malawi, Blantyre, at an altitude of ~200 m above sea level. It has mean annual maximum temperatures between 30–32°C and mean annual minimum temperatures between 18–20°C, and has an annual rainfall of ~800–1 200 mm.

Thyolo is ~39 km from Blantyre at an altitude of ~820 m above sea level. It has mean annual maximum temperatures of ~29°C and mean annual minimum temperatures of ~19°C and has an annual rainfall of ~1 258 mm.

Study seasons and selection of village households

The Chikwawa study was carried out during the dry, rainy and cool seasons from October 2001 to October 2002. Nine villages were sampled during the dry season (October 2001), rainy season between (October 2001 and August 2002 to October 2002) and the cool season (May 2002 to July 2002). The Thyolo study was also carried out during the dry, rainy and cool seasons. Twenty villages were sampled during the dry season (October 2002), rainy season between (August 2002 to October 2002) and the cool season (May 2003 to July 2003).

Permission was sought from relevant authorities to carry out the intended study in Chikwawa and Thyolo Districts. A series of meetings was held with chiefs, the Project Officer, Development Officers, Field Assistants and villagers to explain to them about the intended study in different areas of both Districts. From a list of volunteer village households who owned livestock and wished to participate in the study, 31 livestock owners volunteered to participate in Chikwawa and 34 livestock farmers in Thyolo.

Sample collection

Faecal samples were collected once per season from non-diarrhoeic and diarrhoeic young and adult animals into 50 g capped plastic sample containers (Enterprise Containers, Blantyre, Malawi). Farmers were visited between 05:00 and 09:00 before they released the animals from their kraals for foraging, browsing or grazing. The animals were stimulated to defaecate by making them move about in their kraals. A faecal sample was scooped using a clean wooden applicator stick from the centre of the stool and carefully placed into a clean plastic container which was sealed and identified with a unique sample number. The date of collection, village, sex, age, colour and condition of the animal, macroscopic appearance of the stool (diarrhoeic or formed), and name of the owner of the animal and of the sample collector were entered onto a form. Samples were transported in cool boxes to the laboratory at the University of Malawi, The Polytechnic (UMP), and stored at 4°C until analysed.

Cryptosporidium oocyst detection

A representative portion of faeces (approx. 500 mg) was smeared onto a clean glass slide with a wooden applicator stick, air dried, fixed in methanol for 3 min and subjected to modified Ziehl Neelsen (mZN) staining (Casemore 1991; Smith 1992).

Oocyst abundance

Oocyst abundance was determined by assessing the average number of oocysts seen per ten fields (1+ = <5 oocysts per field; 2+ = >5 oocysts per field; 3+ = >20 oocysts per field). Oocysts were identified using the 40x objective and confirmed under the 100x oil objective.

Confirmation of putative *Cryptosporidium* oocysts

A series of confirmatory procedures were performed at the Scottish Parasite Diagnostic Laboratory (SPDL) on UMP mZN putative *Cryptosporidium* oocyst positive samples obtained from Chikwawa in the rainy season (November 2001 to February 2002). Modified Ziehl Neelsen putative positives were re-examined using auramine phenol (AP) according to Casemore (1991) and Smith (1992) following formol-ether concentration (Allen & Ridley 1970). AP and mZN positive samples were also re-examined using a fluorescein isothiocyanate monoclonal antibody (FITC-C-mAb) (Crypt-a-glo, Waterborne Inc., New Orleans, USA) and the nuclear intercalator 4' 6 diamidino-2-phenyl indole (DAPI) (Grimason, Smith, Parker, Bukhari, Campbell & Robertson 1994; Smith, Campbell, Paton & Nichols 2002). Ten mZN, AP and FITC-C-mAb positive samples were then analysed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) at both an 18S rRNA locus (N-18S rRNA) (Nichols, Campbell & Smith 2003) and the single tube nested *Cryptosporidium* oocyst wall protein (STN-COWP) locus (Homan, Van Gorkom, Kan & Hepener 1999).

Epifluorescence microscopy

Oocysts were visualized by epifluorescence microscopy and their internal morphology assessed using Nomarski differential interference contrast (DIC) microscopy. A blue filter block (excitation 490 nm; emission 510 nm) was used to visualize FITC-C-mAb localization and an ultra-violet (UV) excitation (excitation 355 nm, emission 450 nm) was employed to determine the presence of DAPI-stained sporozoite nuclei. All assessments of the presence of fluorescent nuclei and internal morphology were undertaken at either 400x or 1 000x magnification.

Molecular typing

Oocyst purification and DNA extraction

Cryptosporidium oocyst positive samples were purified by water ether concentration (Bukhari & Smith 1995; Nichols, Moore & Smith 2006) or immunomagnetic separation (IMS, Dynal A.S. Oslo, Norway) according to the manufacturer's instructions. Oocysts were suspended in lysis buffer [50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.5 % sodium dodecyl sulphate (SDS)] and DNA was released from oocysts following 15 cycles of freeze-thawing (freezing in liquid nitrogen for 1 min, followed by thawing at 65 °C for 1 min). Proteinase K (final concentration 200 µg mL⁻¹) was added, and the samples incubated at 55 °C in a water bath, for 3 h. Proteinase K was heat denatured (90 °C, 20 min), samples chilled on ice for 1 min, and centrifuged for 5 min at 10 000 x g (Nichols & Smith 2004). The supernatant (70 µL) was transferred to a clean tube and either used immediately for PCR amplification or stored at -20 °C until used.

PCR protocol

PCR amplifications were performed in Perkin Elmer thermocycler model 480 in 0.5 mL thin-walled tubes. For the direct 18S rRNA PCR reaction volumes of 100 µL consisted of pre-mixed reagents consisting of 200 µM of each of the four dNTP's (Amersham Pharmacia Biotech UK Ltd., Amersham Place, Little Chalfont, Bucks., HP7 9NA, UK), 0.2 µM each of primers CPBDIAGF/R (Cruachem, Todd Campus Acre Road, Glasgow, G20 0UA, UK), bovine serum albumin at final concentration of 400 µg mL⁻¹, MgCl₂ at 3.5 mM, 2.5 U of *Taq* polymerase (Advanced Biotechnologies, Holly Ditch Farm, Mile Elm, Calne, Wiltshire, SN11 0PY, UK) in 1 x PCR buffer IV (Advanced Biotechnologies) and Tween 20 at a final concentration of 2 % to inactivate 0.05 % sodium dodecyl sulphate. Approximately 40 µL of mineral oil was layered on top of each tube containing the dispensed PCR mix and 10 µL of DNA template was pipetted under the oil. Positive controls consisted of *C. parvum* oocyst DNA and negative controls were DNA-free reaction tubes. Samples were subjected to 39 cycles of amplification according to Johnson, Pieniazek, Griffin, Misener & Rose 1995) and the 435 bp PCR product was visualized following ethidium bromide staining of 1.4 % agarose gels (Nichols *et al.* 2003, 2006). PCR protocol for the STN-COWP locus was according to Homan *et al.* (1999) with the inclusion of bovine serum albumin and Tween 20 in the PCR mixture as described above.

Cryptosporidium species identification by PCR-RFLP analysis

Twenty µL of 18S rRNA PCR product were simultaneously digested with 20U of each restriction enzymes *Ase* I (New England Biolabs, 75–77 Knowl Piece, Wilbury Way, Hitchin, Herts, SG4 0TY, UK) and *Dra* I (Invitrogen, 3 Fountain Drive, Inchinnan Business Park, Renfrew, PA4 9RF, UK) in 50 µL of 1 x NE Buffer 3 (New England Biolabs) for 2 h at 37 °C. Ten microliters of PCR product from the STN-COWP assay (Homan *et al.* 1999) was digested with 10 U of restriction enzyme *Taq* I (Invitrogen) according to the supplier's instructions. Undigested controls were run alongside the digested fragments in a 2 % agarose gel at 100 volts for 1–2 h and stained with ethidium bromide (0.5 µg mL⁻¹) incorporated in the gel and the running buffer.

Quality assurance

All diagnostic methods performed at SPDL were conducted in compliance with the Standard Operating Procedures accredited by Clinical Pathology Accreditation (UK) Ltd. and the Drinking Water Inspectorate (Regulatory *Cryptosporidium* Standard Operating Procedures). All methods performed at UMP are accredited at SPDL.

RESULTS

Cryptosporidium in Malawian faecal samples

A total of 1346 faecal samples were analysed. Of these, 905 were from Chikwawa consisting of 559 samples collected in the dry season, 189 in the rainy season and 157 in the cool season, and 441 samples were from Thyolo consisting of 219 samples collected in the dry season, 114 in the rainy season and 108 in the cool season. *Cryptosporidium* oocysts were present in 16.7 % (224/1346) of samples, with a similar rate of occurrence in Chikwawa (7.7 %, 104/1346) and in Thyolo (8.9 %, 120/1346).

The number of animal species sampled and the percentage positivity in the three seasons from both Chikwawa and Thyolo are presented in Table 1. The percentage of samples that was oocyst positive was dependent on season in the two districts sampled, with the highest percentage positives detected in the cool season (31.9 %) in Chikwawa and in the dry season (34.7 %) in Thyolo (Table 2).

The majority of samples were from cattle (61.3 %, 825/1346), with 47.6 % being from cows, 14.2 % from heifers, 7 % from bulls, 1.4 % from oxen (cas-

TABLE 1 Percentage of animals infected with *Cryptosporidium* species oocysts during the cool, dry and rainy seasons in the Chikwawa and Thyolo Districts of Malawi

| Animal species | Chikwawa District | | | | | | Thyolo District | | | | | | | |
|----------------|-------------------|----|------------|----|--------------|----|-----------------|-----|------------|-----|--------------|------|-----|------|
| | Cool season | | Dry season | | Rainy season | | Cool season | | Dry season | | Rainy season | | | |
| | N | n | N | n | N | n | N | n | N | n | N | n | | |
| Cow | 122 | 46 | 389 | 29 | 129 | 10 | 7.8 | 48 | 8 | 89 | 35 | 49 | 14 | 28.6 |
| Goat | 18 | 1 | 41 | 1 | 57 | 0 | 0 | 33 | 1 | 43 | 14 | 33 | 6 | 18.2 |
| Sheep | - | - | - | - | - | - | - | - | - | 5 | 0 | 1 p | 0 | 0 |
| Pig | 17 | 3 | 22 | 4 | 2 | 0 | 0 | 12 | 3 | 25 | 15 | 14 | 5 | 17.9 |
| Rabbit | - | - | - | - | - | - | - | 1 p | 1 | 3 p | 1 | 33.3 | 1 p | 0 |
| Guinea pig | - | - | - | - | - | - | - | - | - | 3 p | 2 | 66.7 | - | - |
| Chicken | - | - | 38 | 4 | - | - | - | 4 | 1 | 13 | 5 | 38.5 | 4 | 50 |
| Guinea-fowl | - | - | 16 | 2 | - | - | - | - | - | 2 | 0 | 0 | 1 p | 0 |
| Duck | - | - | 16 | 2 | - | - | - | 8 | 0 | 11 | 3 | 27.3 | 3 | 0 |
| Turkey | - | - | - | - | - | - | - | 1 | 0 | 7 | 1 | 14.3 | 6 | 16.7 |
| Dove | - | - | 38 | 2 | - | - | - | 1 | 0 | 18 | 0 | 0 | 2 | 0 |

N = number of animals examined

n = number of animals infected

p = pooled sample

% = percentage of animals infected

TABLE 2 Percentage of animals excreting *Cryptosporidium* species oocysts during the dry, rainy and cool seasons in the Chikwawa and Thyolo Districts of Malawi

| Season | Chikwawa | | Thyolo | |
|--------|--------------|------------------|--------------|------------------|
| | No. examined | No. infected (%) | No. examined | No. infected (%) |
| Dry | 559 | 44 (7.9) | 219 | 76 (34.7) |
| Rainy | 189 | 10 (5.3) | 114 | 28 (24.6) |
| Cool | 157 | 50 (31.9) | 108 | 14 (13) |

TABLE 3 Percentage of cattle excreting *Cryptosporidium* species oocysts by age and season in the Chikwawa and Thyolo Districts of Malawi

| Age | Thyolo | | | | | | | | | | | | | | | | | |
|---------|-------------|----|------------|-----|--------------|------|-------------|----|------------|----|--------------|------|----|----|------|----|---|------|
| | Chikwawa | | | | Thyolo | | | | Thyolo | | | | | | | | | |
| | Cool season | | Dry season | | Rainy season | | Cool season | | Dry season | | Rainy season | | | | | | | |
| | N | n | % | N | n | % | N | n | % | N | n | % | | | | | | |
| Cows | 60 | 22 | 36.7 | 156 | 10 | 6.4 | 69 | 5* | 7.2 | 28 | 4 | 14.3 | 51 | 16 | 31.4 | 29 | 7 | 23.3 |
| Heifers | 25 | 6 | 24 | 52 | 5 | 9.6 | 22 | 3* | 1.4 | 7 | 1 | 14.3 | 4 | 2 | 50 | 7 | 5 | 71.4 |
| Bulls | 11 | 4 | 36.4 | 26 | 3 | 11.5 | 12 | 0* | 0 | - | - | - | 4 | 3 | 75 | 5 | 1 | 20 |
| Oxen | 2 | 2 | 50 | 6 | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | - |
| Calves | 24 | 12 | 50 | 147 | 11 | 7.5 | 26 | 0* | 0 | 11 | 3 | 27.3 | 30 | 14 | 46.7 | 8 | 1 | 12.5 |

N = number of animals examined

n = number of animals infected

% = percentage of animals infected

* = values exclude two animals of unknown age

TABLE 4 Percentage of cattle excreting *Cryptosporidium* species oocysts by age in the Chikwawa and Thyolo Districts of Malawi

| Age | Chikwawa | | Thyolo | |
|--------------|--------------|------------------|--------------|------------------|
| | No. examined | No. infected (%) | No. examined | No. infected (%) |
| Adult cattle | 441 | 60 (13.6) | 135 | 39 (28.9) |
| Calves | 197 | 23 (11.7) | 49 | 18 (36.7) |

TABLE 5 SPDL quality assurance samples obtained from bovines in Chikwawa during the 2001/2002 collection

| Village | TA | Farmer | No. | Age | Breed | SC | OA | Direct 18S rRNA | STN-COWP | <i>Cryptosporidium</i> species |
|--------------|---------|-----------|------|--------|-------|----|------|-----------------|----------|--|
| Chikambi III | Kasisi | Shuva | 12 a | Cow | MZ | D | 2+ | Negative | Negative | |
| Chikambi III | Kasisi | Shuva | 12 c | Cow | MZ | D | 1-2+ | Negative | Negative | |
| Mvula | Katunga | Chidzenga | 13 c | Heifer | MZ | D | 1-2+ | Negative | Negative | |
| Mvula | Katunga | Chidzenga | 13 d | Heifer | MZ | D | 1+ | Positive | Negative | Mixed infection of <i>C. parvum</i> , <i>C. hominis</i> , <i>C. bovis</i> or <i>C. ryanae</i> and <i>C. andersoni</i> or <i>C. muris</i> |
| Mvula | Katunga | Chidzenga | 13 e | Cow | MZ | D | 2+ | Negative | Negative | |
| Mvula | Katunga | Chidzenga | 13 l | Heifer | MZ | D | 1-2+ | Positive | Positive | <i>C. parvum</i> |
| Mvula | Katunga | Chidzenga | 13 j | Cow | MZ | D | 1-2+ | Negative | Negative | |
| Mvula | Katunga | Chidzenga | 13 m | Cow | MZ | D | 1-2+ | Positive | Negative | <i>C. parvum</i> , <i>C. hominis</i> , <i>C. bovis</i> and/or <i>C. ryanae</i> |
| Mbendelana | Kasisi | Zamala | 1 k | Nd | MZ | D | 1+ | Negative | Negative | |
| Mbendelana | Kasisi | Zamala | 1 t | Nd | MZ | D | 1+ | Positive | Negative | <i>C. parvum</i> , <i>C. hominis</i> , <i>C. bovis</i> and/or <i>C. ryanae</i> |

TA = Traditional Authority

MZ = Malawi zebu

SC = stool consistency

No. = specimen number

D = diarrhoeic

OA = oocyst abundance (1+ = < 5 oocysts/field; 2+ = > 5 oocysts/field at a magnification of X 20)

Nd = no data

COWP = single tube nested PCR for the COWP gene fragment

trated males) and 29.8 % from calves (<6 months old). Stool samples from other mammalian livestock (goats [$n = 225$], pigs [$n = 92$] and sheep [$n = 6$]), as well as rabbits, guinea pigs, chicken, ducks, turkeys, doves and guinea fowls) were also analysed ($n = 197$; Table 1).

In Chikwawa, between 7.8 % and 37.7 % of cattle samples contained oocysts, dependent on season, with the highest percentage of infections occurring in the cool season and the lowest in the rainy season (Table 1). In Thyolo, between 3 % and 32.6 % of cattle samples contained oocysts, dependent on season, with the highest percentage of infections occurring in the dry season and the lowest in the cool season (Table 1). In Chikwawa, between 0 % and 5.6 % of goat samples contained oocysts, with the highest percentage of infections again occurring in the cool season and the lowest in the rainy season (Table 1). In Thyolo, between 3 % and 32.6 % of goat samples contained oocysts, with the highest percentage of infections occurring in the dry season and the lowest in the cool season (Table 1). In Chikwawa, the highest percentage of infections in pigs were detected in the dry season, but infections in the cool season were similar (17.7 %), whereas in Thyolo, infections occurred in all three seasons (17.9 % in the rainy season, 25 % in the cool season and 60 % in the dry season) (Table 1).

Cryptosporidium infections in rabbits, guinea pigs, chicken, ducks, turkeys, doves and guinea fowls were also detected, and while the data indicate that infections were detected in these hosts, the data are sparse and do not cover all seasons analysed. Based on mZN positivity, Table 2 identifies the numbers and percentages of stools containing oocysts in our samples, over the three seasons analysed and serves as an indicator of the prevalence of *Cryptosporidium* in the Chikwawa and Thyolo Districts of Malawi. *Cryptosporidium* oocysts were more likely to be detected in the cool season in Chikwawa and in the dry season in Thyolo whereas oocysts were least likely to be detected in the dry season in Chikwawa and in the cool season in Thyolo. The age-based breakdown of *Cryptosporidium* oocyst positive samples from cattle is presented in Tables 3 and 4. We detected *Cryptosporidium* oocysts during all three seasons in the Chikwawa and Thyolo districts of Malawi. In Chikwawa, 13.6 % of adult cattle and 11.7 % of calves were infected, compared to 28.9 % of adult cattle and 36.7 % of calves in Thyolo (Table 4). Season influenced *Cryptosporidium* occurrence in adult cattle and calves in Chikwawa and Thyolo. The highest percentages of oocyst positivity

occurred in both older cattle and calves in the cool season in Chikwawa and in the dry season in Thyolo with nearly 50 % of calves being infected (Table 3).

Quality assurance at SPDL on UMP mZN putative *Cryptosporidium* oocyst positive samples obtained from cattle in Chikwawa

Ten diarrhoeic faecal samples collected from Chikwawa (November 2001 to February 2002) which were mZN positive at UMP were also AP and IFAT positive at SPDL, indicating that the SPDL mZN Standard Operating Procedure used at UMP could detect *Cryptosporidium* spp. oocysts. These ten diarrhoeic samples were subjected to PCR-RFLP. Three (30 %) had an oocyst abundance of 1+, five (50 %) had an oocyst abundance of 1–2+ and two (20 %) had an oocyst abundance of 2+ (Table 5). Four oocyst positive samples (two from heifers, one from a cow and 1 unknown) were amplified using the N-18SrRNA and STN-COWP assays. At the 18S rRNA locus, following *Ase* I /*Dra*I digestion, all four samples showed a RFLP pattern that corresponds to *C. parvum*, *C. hominis*, *C. bovis* or *C. ryanae* (previously, *Cryptosporidium* deer-like genotype), or a mixture of them. Only one of the four 18S rRNA positive samples amplified at the STN-COWP locus and the RFLP pattern indicated the presence of *C. parvum* DNA.

DISCUSSION

This is the first study to report *Cryptosporidium* infection in Malawian livestock and other non-human hosts and is of potential veterinary and public health importance. It included most of the animals that the volunteer livestock owners possessed, and, as such, should also offer useful *Cryptosporidium* point prevalence data for livestock (primarily cattle) in the Chikwawa and Thyolo Districts of Malawi. Elsewhere, *Cryptosporidium* spp. have been shown to be a significant cause of morbidity in animals and humans, particularly among neonates and immunocompromised hosts. We detected *Cryptosporidium* oocysts in 16.7 % of samples, with a similar rate of occurrence in both Chikwawa and Thyolo (7.7 % and 8.9 %), respectively. With the exception of the ten quality assurance samples analysed at SPDL, all positive samples were detected using mZN at UMP. As all ten UMP mZN positive, quality assurance samples were also oocyst positive by AP and IFAT, we can be assured that the SPDL mZN Standard Operating Procedure used at UMP detects *Cryptosporidium* spp. oocysts.

The absence of detectable oocysts in our unconcentrated samples (October 2001 [dry season], November 2001 to February 2003 [rainy season]) does not signify that the animals tested did not harbour *Cryptosporidium* infection as our detection methods are insensitive. Using unconcentrated faecal smears, Anusz, Mason, Riggs & Perryman (1990) reported a detection limit of 10^6 oocysts mL^{-1} of faeces using Kinyoun staining. Oocyst concentration methods can increase the sensitivity of detection. Between 1×10^4 and 5×10^4 oocysts g^{-1} of unconcentrated stool are necessary to obtain a 100 % detection efficiency using the Kinyoun staining method (Weber, Bryan, Bishop, Wahlquist, Sullivan & Juranek 1991). Variations in faecal consistency influence the ease of detection, with oocysts being more easily detected in concentrates made from watery, diarrhoeal specimens than from formed stool specimens (Weber *et al.* 1991). Webster, Smith, Giles, Dawson & Robertson (1996) reported that oocysts were not detected in bovine faecal samples seeded with 10^4 *C. parvum* oocysts g^{-1} following formol ether concentration and examined using AP or IFAT, and that sucrose or salt flotation was required to increase the threshold of detection to $4\text{--}6 \times 10^3$ oocysts g^{-1} using AP or IFAT.

With the exception of the small numbers of sheep samples analysed ($n = 6$), *Cryptosporidium* spp. oocysts were detected in all other host species (Table 1), indicating the commonness of this parasite in Malawi. However, conventional staining methods (e.g. mZN, AP, IFAT) cannot determine the species/genotype of *Cryptosporidium* infecting these hosts. The majority of samples analysed were from cattle (61.3 %), with 29.8 % sampled from calves (< 6 months old). Calves have been implicated in the zoonotic transmission of cryptosporidiosis, frequently (Mallon, MacLeod, Wastling, Smith, Reilly & Tait 2003a; Mallon, MacLeod, Wastling, Smith, & Tait 2003b; Geurden, Berkvens, Martens, Casaert, Vercruysse & Claerebout 2007; Xiao & Feng 2008).

Cattle can be infected with *C. parvum*, *C. bovis*, *C. ryanae* (previously, *Cryptosporidium* deer-like genotype), *C. andersoni* and *C. suis* (Fayer 2008) and cryptosporidiosis, due to *C. parvum* or *C. andersoni*, is a cause of production losses in cattle (Esteban & Anderson 1995; Smith 2008). Neonatal cattle and sheep act as reservoirs for the zoonotic transmission of *C. parvum* (Smith *et al.* 2007). Whereas calves < 6 months old are most likely to harbour *C. parvum*, older cattle are more likely to harbour the host adapted *C. bovis* and *C. ryanae* (Feng, Ortega, He, Das, Zhang, Fayer, Gatei, Cama & Xiao 2007).

Of the other livestock investigated, goats are infected with *C. parvum* (Fayer 2008), the *C. bovis*-like genotype and a novel *Cryptosporidium* genotype (now *C. xiaoi*, Fayer & Santin 2009) (Karanis, Plutzer, Halim, Igori, Nagasawa, Ongerth & Liqing 2007), pigs with *C. suis*, *Cryptosporidium* pig genotype II and *C. parvum* (Fayer, 2008; Zintl, Neville, Maguire, Fanning, Mulcahy, Smith & De Waal 2007) and sheep with *C. parvum*, *Cryptosporidium* cervine genotypes 1–3 and *Cryptosporidium* sheep novel genotypes (Fayer 2008), *C. hominis*, *C. andersoni*, marsupial genotype, pig II genotype, and a novel unidentified genotype (Ryan, Bath, Robertson, Read, Elliot, McInnes, Traub & Besier 2005), a novel *C. bovis*-like genotype (now *C. xiaoi*, Fayer & Santin 2009; 99.9 % sequence similarity with *C. bovis*) (Santin, Trout & Fayer 2007).

In addition to the likelihood of some of these *Cryptosporidium* species causing disease in their major hosts, a zoonotic potential also exists for those animals infected with human infectious species, particularly, but not exclusively, those infected with *C. parvum*.

We detected *Cryptosporidium* oocysts during all three seasons in the Chikwawa and Thyolo Districts of Malawi. In Chikwawa, 13.6 % of adult cattle and 11.7 % of calves were infected, compared to 28.9 % of adult cattle and 36.7 % of calves in Thyolo (Table 4). Season influenced *Cryptosporidium* occurrence in adult cattle and calves in Chikwawa and Thyolo. Chikwawa experiences higher temperatures and lower annual rainfall than Thyolo, and temperature influences oocyst survival.

Oocysts remain infectious for up to 3 months when stored in water at ambient (20–30 °C) temperatures (Fayer, Trout & Jenkins 1998) but higher temperatures and increased solar radiation increase *Cryptosporidium* oocyst inactivation in the environment (Fujino, Matsui, Kobayashi, Haruki, Yoshino, Kajima & Tsui 2002; King, Daminato, Fanok & Monis 2008). King *et al.* (2008) found that solar UV can rapidly inactivate *C. parvum* in environmental waters, and determined that solar radiation was a critical process affecting oocyst survival in the environment. Animal management systems also differ in Chikwawa and Thyolo. In Chikwawa District most cattle are beef cattle and free range grazers, whereas, in Thyolo, the majority of cattle are dairy cattle and are housed and fed using the cut and carry method. Increased milk production demands and scarcity of feed can cause body condition loss and increased susceptibility to infectious diseases. These season-

al, temperature and animal management regime differences warrant further investigation.

As age influences the species of *Cryptosporidium* infecting bovines, with calves less than 6 months old being most likely to harbour the zoonotic species, *C. parvum* (Feng *et al.* 2007), there should be a greater potential for the zoonotic spread of *C. parvum* in the cool season in Chikwawa and in the dry season in Thyolo, where infection in calves was highest (~50%; Table 3). Furthermore, the zoonotic potential might be greater in Thyolo than in Chikwawa, as 36.7% of calves were infected in the former compared with 11.7% in the latter district (Table 4).

Cryptosporidium is a common infection of bovines worldwide. Kaminjolo, Adesiyun, Loregnard & Kitson-Piggot (1993) reported a prevalence of 8.7% in calves in Trinidad and Tobago, and, based on single samplings, *Cryptosporidium* prevalence rates in calves ranging from 5.6–79.0% have been reported from the USA (Sobieh, Tacal, Wilcke, Lawrence & El-Ahraf 1987; Ongerth & Stibbs 1989). Mtambo *et al.* (1997) stated that 5.3% of bovine faecal samples in Tanzanian farms contained oocysts and that *Cryptosporidium* prevalence was higher in calves < 3 months of age compared to weaned calves and adults. Huetink, Giessen, Noordhuizen & Ploeger (2001) reported *Cryptosporidium* prevalence to be 2.4–22.2% in a dairy cattle herd in The Netherlands. A Spanish study reported a *C. parvum* prevalence of 7.7% in fattening calves and heifers between 4–24 months old and 17.8% in adult cattle (Quilez, Sanchez-Acedo, del Cacho, Clavel & Causape 1996). *Cryptosporidium* prevalences of 10.5% in dairy cows, 8.7% in heifers and 28.8% in beef cattle (Fayer, Trout, Graczyk & Lewis 2000), 70.2% in asymptomatic adult cattle (Lorenzo-Lorenzo, Ares-Mazas & Villacorta-Martinez de Maturana 1993), 17.8% in asymptomatic cattle over 2 years of age (Quilez *et al.* 1996), 81% in 2–36 day-old calves, 15% in 1.5–4.5 month-old post-weaned calves, 0% in 20–24 month-old heifers (Ares-Mazas, Fernandez-da Ponte, Vergara-Castiblanco, Frieve-Santos, Quilez-Cinca, Causape-Valenzuela & Sanchez-Acedo 1999), 62.4% in apparently health adult cattle (Scott, Smith, Mtambo & Gibbs 1995) and 19% in adult dairy cows (Bukhari & Smith 1996) have been documented. These findings are in agreement with the results of this study.

This study clearly demonstrates the presence of *Cryptosporidium* infections in a variety of avian and mammalian hosts in Malawi, indicating the potential role of such hosts as reservoirs of infectious parasites, and in particular infections in post-weaned

and adult cattle. Although we did not use molecular typing tools in our survey, we did use them for quality assurance. Four of the ten mZN, AP and IFAT positive samples used for quality assurance at SPDL were amplified by PCR using the direct 18S rRNA gene locus, but only one oocyst positive sample amplified at the STN-COWP locus. The direct 18S rRNA gene locus can determine all *Cryptosporidium* species after sequencing, while the STN-COWP locus can identify *C. parvum*, *C. hominis* and *C. meleagridis* by RFLP (Jiang & Xiao 2003). Although each oocyst contains 20 copies of the 18S rRNA gene, the direct 18S rRNA PCR was only marginally sensitive with these ten oocyst positive slides. The expansion of valid *Cryptosporidium* species in the last 5 years (Smith *et al.* 2007) identifies that RFLP analysis is becoming less useful currently, as *Ase I* and *DraI* digestion of the 18S rRNA amplicon cannot discriminate between *C. parvum*, *C. hominis*, *C. bovis* and *C. ryanae*. The *C. parvum* RFLP pattern was identified from the only oocyst positive sample that amplified at the STN-COWP single copy gene locus. The low PCR positivity rate using these loci may be due to co-extraction of inhibitors with DNA, the amount of DNA template extracted and/or the absence of nuclear material within the oocysts. Most of our microscopy positive samples had low oocyst abundances (highest oocyst abundance = 2+), possibly indicating either the tailing off of cryptosporidiosis or asymptomatic infection and the low sensitivity of mZN. *Cryptosporidium parvum* was unambiguously detected in one (10%) sample (heifer; Table 5) establishing that *C. parvum* infection occurs in Malawian cattle. Since *C. parvum* can infect susceptible human and non-human hosts, this species has both veterinary and public health significance in Malawi.

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