NAD1 RFLP-PCR FOR DETECTING ECHINOCOCCUS PREVALENCE IN CARNIVORES IN WHITE NILE AREA, SUDAN. A PILOT STUDY

Makky, A[1], Thomas, R[2]; Kern, P[3]; Omer, Rihab A.[4,5]

1. Rabak Veterinary Research Laboratories, Animal Resources Research Cooperation, Sudan
2. Department of Parasitology, Institute of Zoology, University of Hohenheim, Stuttagrt, Germany.
3. Section of Infectology and Clinical Immunology, Department of Internal MedicineII, University of Ulm, Germany
4. Central Veterinary Research Laboratories, Khartoum, Sudan
5. Department of Parasitology, Faculty of Veterinary Medicine, University of Leipzig, Leipzig, Germany

المستخلص

فحصت 158 عينة من البراز للكشف عن بيض أنواع المشوكات المختلفة، 150 عينة كانت من الكلاب الضالة، 5 عينات من الثعالب و 3 من الكلاب البرية في مناطق مختلفة بولاية النيل الأبيض في السودان خلال الفترة من أبريل 2009 إلى سبتمبر 2010. تم تحديد نوع البراز مبدئياً بواسطة الشكل، اللون، الرائحة ووجود العلامات الحقلية. أوضحت النتائج وجود بيض الـTaeniid في في 73 من الكلاب، 3 من الثعالب وو كلب بري واحد. تم عزل الحامض النووي من 311 من البيض: 292 من الكلاب الضالة، 15 من الثعالب و 4 من الكلاب البرية.

تم إستخدام تقنية RELP-PCR لتحليل الجين nad 1 بالميتوكوندريا لتحديد النوع. وتم الحصول على منتجات التضخيم في 69.8% من العينات الموجبة بالكلاب الضالة (73/51) و 33% من العينات الموجبة بالثعالب (3/1) ولم توجد في عينات الكلاب البرية. تم التأكيد على هذه النتائج من تسلسل الحمض النووي بالميتوكوندريا في مجموعة فرعية من 5 عينات من الكلاب وعينة واحدة من الثعالب. أوضحت هذه النتائج أن نوع *Echinococcus canadensis* هو النوع السائد في هذه الحيوانات في تلك المنطقة كما وصى بإجراء مزيد من التجار والدراسات في عدد أكبر من الحيوانات.

Corresponding author: Rihab A. Omer. Address: An den Tierkliniken 35, 04103 Leipzig, Germany, Telephone: 00493419738475, Fax: 00493419738095, Email: omer@vetmed.uni-leipzig.de.

Abstract

One hundred fifty eight faecal samples were examined: 150 from stray dogs, 5 from foxes (*Vulpes pallida*) and 3 from Wild dogs (*Lycaon pictus*) in different localities within the White Nile Province in Sudan in the period between April 2009 and September 2010 for eggs of *Echinococcus* species. The origin of the faeces was tentatively determined by shape, colour, odour and the presence of field signs. Taeniid eggs were demonstrated in 73 dogs, 3 foxes and 1 wild dog. DNA was isolated from 311 eggs (292 form dogs, 15 from foxes and 4 form wild dogs) for species identification. A previously described restriction fragment length polymorphism (RFLP) - PCR of the mitochondrial nad1 gene was used for species identification. Amplification products were obtained from 51 of the 73 (69.8%).taeniid egg-positive samples obtained from stray dogs, 1/3 (33%) of foxes and none (0%) of wild dogs. *E.canadensis* (G6) was identified in 100% of those samples. This result was further confirmed by mitochondrial DNA sequencing of a subset of 5 samples of dog and one sample of fox.

It was concluded that *E. canadensis (G6)* appear to be the predominant strain in carnivores in that areas. Further studies including more number of carnivores are highly recommended.

Key words: Echinococcus, Carnivores, Prevalence, genotyping, Sudan

Introduction

Echinococcus is a cestode that requires two mammalian hosts for completing its life cycle. However, compared to members of the genus Taenia, Echinococcus exhibits marked difference not only in morphology but also in lower host specificity and much greater reproductive potential for the metacestode (Thompson et al. 1984). The parasite is transmitted between domestic dogs or other carnivores which harbour the adult tapeworm and herbivorous, which act as intermediate host for the cystic larval stage. Cystic Echinococcosis (CE) is a zoonotic disease with a wide geographical distribution, with emerging and re-emerging regions mainly in Central Europe and China (Eckert and Deplazes 2004). Echinococcus species have high biotic potential making infected dogs as the definitive hosts able to excrete a great number of segments full of egg with their faces contaminating the soil and spreading the infection (Gemmell et al. 1990; Benito et al. 2006). Due to that detection of infection in the definitive host has a great importance for epidemiological and ecological studies and also designing an effective strategy for control programs (Dalimi et al. 2002). Many livestock rearing areas of northern and eastern Africa have particularly high prevalences of Echinococcosis, both in human populations and livestock. Compared with other parts of the world, the epidemiology of cystic Echinococcosis in sub-Saharan Africa is rather poorly understood. In Sudan, it was noticed over years that hydatid disease is highly prevalent in most of the animals slaughtered for meat consumption with the camels having the highest prevalence rates (Omer et al. 2010b).In central Sudan, domestic livestock and dogs are frequently affected (El-Khawad et al. 1979; Saad and Magzoub, 1986). In a recent survey in Tamboul and Rofaa cities in central Sudan, the prevalence of echinoccosis in stray dogs was 53% (35/66) and 44% (8/18) respectively (Omer et al. 2011). In humans, the disease seems to occur rather sporadically, except in the extreme southeast of Equatoria province close to the borders with Kenya and Ethiopia. Prevalence rates based on ultrasound surveys can reach 3.5% e.g. with the Taposa people, but the focus does not seem to extend into the western parts of southern Sudan (Magambo et al. 1996; Magambo et al. 1998). It is highly suggestive that this situation is caused by the presence of the sheep strain (G1) or related genotypes in that area which opposed by higher frequency of E. canadensis G6 in other areas in the Sudan (Omer et al. 2010a). Scattered

surveys have been done to investigate presence of E. granulosus on dogs in different parts of the Sudan and also to describe the life cycle pattern in dogs infected under laboratory conditions (Saad, 1986). The current study-a pilot survey of wild carnivores and stray dogs in White Nile area for Echinococcus and subsequent identification of its species was conducted between 2009 and 2010. The area is characterized by large number of livestock specially sheep, many semi stray (home dogs, but spending the day roaming in the streets and near slaughterhouses) and some wild dogs and foxes coming sometimes near houses and farms, all are factors which enhance the presence and transmission of an important neglected zoonotic disease. A view of taking more comprehensive studies/surveys in the future is planned.

Materials and Methods

Study area

The stray dogs and wild carnivores that were investigated came from different areas and villages in the White Nile State. White Nile State is located in the centre of the Sudan. It is delimited by Khartoum state in the north, North Kordofan State in the west, South Kordofan and the Upper Nile State in the Southeastern and El-Gazira and Sinnar state in the east Fig.1. It has an area of 39701 Km^2 and a total population of 1,726,356 working mostly on agriculture, livestock's rearing. Animal recourses in the area are estimated by 6298095 heads (20155 camels, 2506950 cattle, 1801504 sheep and 1788086 goats).

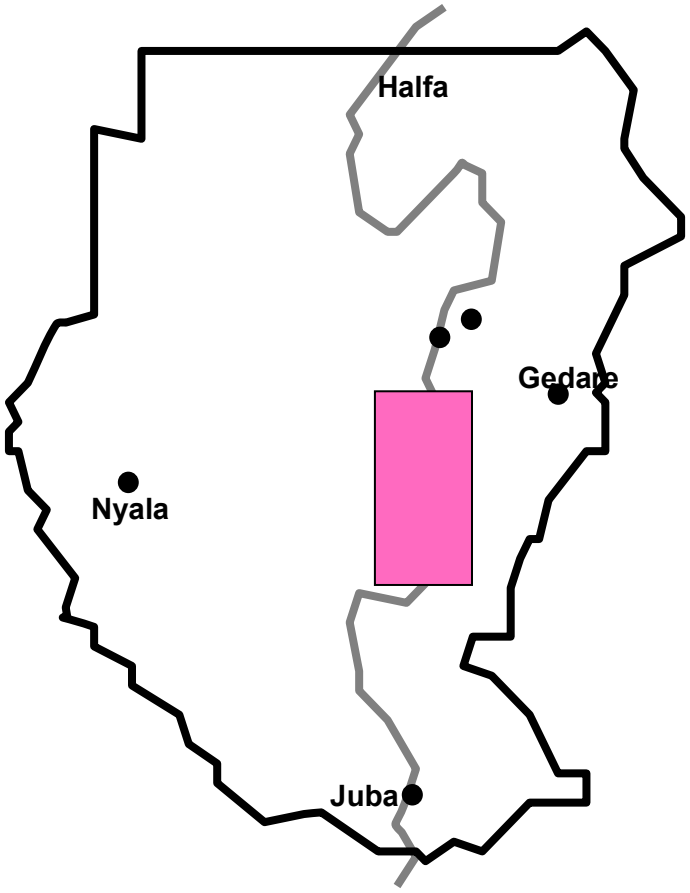

Fig. 1: Study area

Animals and samples

One hundred fifty eight faecal samples from stray dogs (150), foxes (*Vulpes pallida*) (5) and Wild dogs (*Lycaon pictus*) (3) were used in this study. These samples were identified by the experienced technicians who collected the samples recognized the species of mammal that had excreted each sample, based particularly on the footprints or tracks left by carnivores on the soil, the morphology and colour of the faeces, and the defecation site. Some of the faeces were also collected immediately after defecation.

Preparation and investigation of the faecal samples

All faecal samples obtained during the study were frozen at -80ºC for 2 weeks to kill any infectious eggs of Echinococcus spp.

Microscopic examination and DNA amplification

Taeniid eggs were recovered from faces by zinc chloride flotation (Mathis et al. 1996) and then suspended in 10mM Tris-HCl , ph 7.5 containing 1mM EDTA (TE buffer). To reduce the risk of mixed parasitic infection(Muller-Graf 1995), individual eggs were isolated using a capillary pipette. As reported previously (Nakao et al. 2003), a single egg was lysed in 10µl of 0.02 N NaoH at 95ºC for 10 min and used directly as a template for PCR. Template preparations and PCR for the eggs were performed using different rooms, divided reagents and individual pipettes to avoid DNA contamination (Huttner et al. 2008).

RFLP-PCR of the nad1 gene

Identification and characterization of genotypes and species of Echinococcus was done using a new method for differentiation of the known Echinococcus species which are endemic in Africa (Huttner et al. 2009b). This method is based on the amplification of ~ 1075 bp -long fragment including the complete NADH dehydrogenase subunit 1 (nad1) gene with subsequent digestion of the amplification products with the restriction enzyme HphI. For amplification of taeniid egg DNA a nested PCR was performed because of the minute amount of DNA in single taeniid eggs (Rishi and McManus 1987) using the nad1 primers. In the first PCR the primer pairs nadA for: 5' TGT TTT TGA GAT CAG TTC GGT GTG 3'and nadC rev: 5' CAT AAT CAA ACG GAG TAC GAT TAG 3' were used whereas in the primer pairs nadB for: 5' CAG TTC GGT GTG CTT TTG GGT CTG 3' and nadD rev: 5' GAG TAC GAT TAG TCT CAC ACA GCA 3' were used in a nested PCR). The external and internal primers for these mitochondrial genes were designed from the conserved regions of *E. granulosus* mtDNA (database Accession No. AF297617). Reaction mixtures and amplification conditions which were described previously (Hüttner et al., 2008). In brief, 1 µl of egg was added to a reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM of MgCl2, 200 µM of each dNTP, 12.5 pmol of each primer and 1.25 units of Ampli-Taq Polymerase (Applied Biosystems) and amplification was done for 35 cycles (start denaturation for 5 min at 94°C, denaturation for 30 s at 94°C annealing for 30 s at 55°C, elongation for 1 min at 72°C and final elongation for 5 min at 72°C). To save material and time, the PCR products of two samples were digested in

one digestion mixture. The mixture contained 10 µl PCR product of each sample, 8 µl H_2O, 2 µl digestion buffer provided by the manufacturer and 0.5 µl of the restriction enzyme HphI (Fermentas). PCR products were digested for 6 h at 37ºC and the fragments were separated on a 3% ethidium bromide-stained agarose gel. If one uniform pattern was visible, both samples were regarded as the same species. In cases of a combined banding pattern, both samples were digested separately again to allocate each sample to the correct species.

Mitochondrial gene sequencing

A total of 6 (5 of dogs and one of fox) samples was sequenced to determine the intraspecific genotype. Sequencing was done for the partial mitochondrial cox1 gene with primer pair 2575 and 3021 (Bowles et al. 1992) and nad1 gene using primer pair JB11 and JB12 (Bowles and McManus, 1993b). PCR products were purified over Qiaquick columns and cycle sequencing was done as described in Dinkel et al. (2004) on the Gene Amp 2400 (Perkin Elmer) using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) for 25 cycles (denaturation for 10 s at 94°C and annealing for 4 min at 60°C). Electrophoresis was performed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) and nucleotide sequence analysis was done using the BLAST programs and databases of the National Center for Biotechnology Information.

Results

Microscopic examination for the presence of Taeniid eggs

Taeniiade eggs were detected in 73 stray dogs, 3 foxes and 1. Average number of eggs/1gm faecal sample/animal was 3,2, 4,4 and 1,2 in stray dogs, foxes and wild dogs respectively (table1, fig. 2 and fig.3).

Table1: Summary of the faecal microscopically examination results.

Species	**Total number examined**	**Number of samples with *Taeniia* eggs**	**Average number of eggs/animal**	**Prevalence Percentage of *Taeniia* spp. possibly icluding *Echinococcus* spp**
Stray dogs	150	73	3.2	48.6%
Foxes (*Vulpes pallida*)	5	3	4.4	60%
Wild dogs (*Lycaon pictus*)	3	1	1.2	33.3%

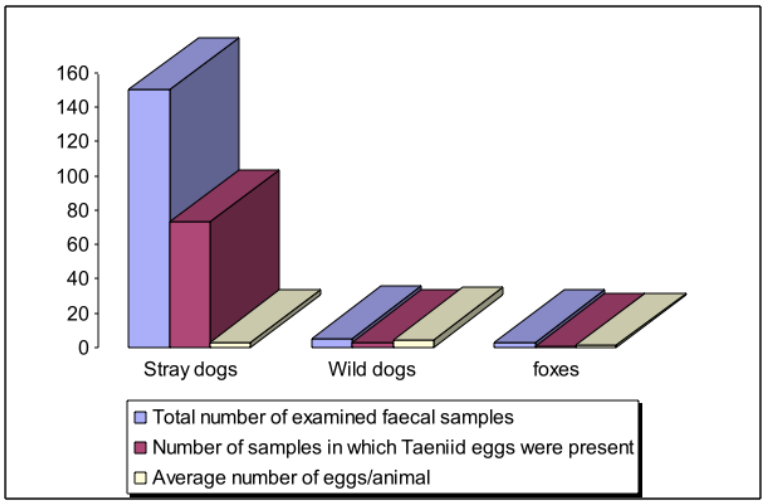

Fig. 2: Presentation of the number of examined carnivores, number of carnivores' with taeniid eggs and average number of eggs in each animal.

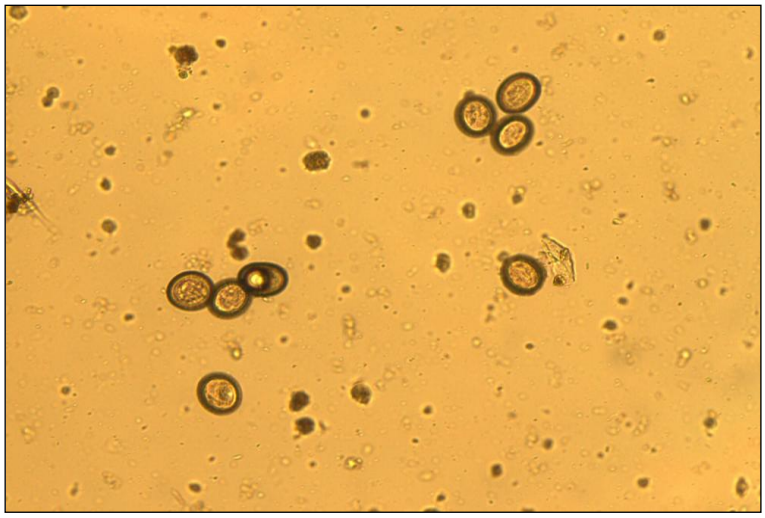

Fig. 3: Taenia egg in a fecal sample of dog origin.

Genetic identification

DNA was isolated from a total of 311 eggs of (292: stray dog, 15: foxes and 4: Wild dogs). From these amplification products were obtained from51 stray dogs, 1 fox and none from Wild dogs (table 2). Enzymatic digestion with Hph1 enzyme shown a similar digestion pattern to *E.canadensis* (Fig. 4) . Results of sequencing showed 100% homology with the 'camel strain' G6 of *E. canadensis* when compared with data on GenBank®. (Accession No. 208063).

Table 2: Summary of the genetical examination results

Species	Total number of recovered taeniid eggs	Nr. and prevalence of animals from which eggs with amplification products were obtained	Strain identification	Nr. samples confirmed by mitochondrial DNA sequencing
Stray dogs	292	51 (34%)	*E.canadensis*	5
Foxes (*Vulpes pallida*)	15	1 (20%)	*E.canadensis*	1
Wild dogs (*Lycaon pictus*)	4	0 (0%)	*E.canadensis*	0

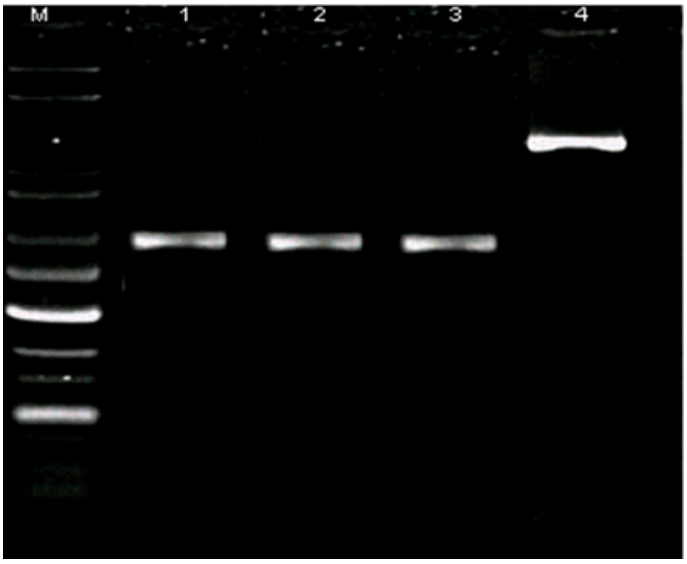

Fig. 4: RFLP- PCR of the nad1 gene with Hph1. Echinococcus canadensis in stray dogs and foxes (*Vulpes pallida*). M: Molecular weight marker, 1-2: Taenia eggs from stray dogs, 3: Taenia egg from fox (*Vulpes pallida*), 4: undigested PCR products.

Discussion

Dogs and other suitable canids (dingos, wolves and foxes) are the major definitive hosts for *E. granulosus*, the cause of unilocular hydatid disease (cystic Echinococcosis) in livestock and humans. The disease is regarded as one of the most important and globally widespread helminth zoonoses. In sub-Saharan Africa, several wild carnivores have been found infected with *E. granulosus* infections, including golden jackals in Chad and Kenya (Troncy and Graber 1969; Macpherson et al. 1983) and blacked jackals (*Ca.mesomelas*) in Kenya (Macpherson et al. 1986). This parasite has also been reported to infect spotted hyaena (*Crocuta crocuta*) in Kenya (NELSON and RAUSCH 1963), and the Cape silver fox (V. chama) in South Africa (Verster and Collins 1966). In a more recent survey from Africa, *E. feldis* was demonstrated in 35 of 47 samples of lions and one of five samples of spotted hyenas (Huttner et al. 2009a). In Sudan, CE was shown to occur in a wide range of intermediate hosts, with the camel being the most important intermediate host in terms of both prevalence and cyst fertility (Omer et al. 2010). Moreover the *E. canadensis* G6 which is the camel strain was found as the much abundant cause of infection in both herbivores and humans which unlikely to many other African countries, some of which has borders with the Sudan, where the *E.granulosus sensu stricto*, sheep strain is responsible for most of the infections in animals and humans.

In the current study, the study area is located in the west central part of the Sudan and characterized by a large number of livestock used for meat consumption. Home slaughtering is a adopted on a large scale, stray and semi stray dogs are very much abundant. Some Wild dogs and foxes start getting very near from the areas which are inhabited by people seeking food. All these factors can help making a suitable environment for the transmission and maintenance of *Echinococcus* species. In a recent survey for the prevalence of CE in herbivores intermediate hosts in the same study area (Makky et al. unpublished), the disease was prevalent in 50%(26/55), 1.6% (26/1560), 00.9% (49/5520) and 0.3% (2/730) camel, cattle, sheep and goats respectively. Curiously, it is important to add that fertility rates of 80% and 23% were reported from camel and cattle opposing lower fertilities of 3% and 0% in sheep and goats in the same study area. As relative frequency of fertile cysts from each of these species available after slaughter has to be considered rather than

prevalence we can come to the conclusion that camel and cattle are the most important species for maintaining the cycle of Echinococcus in our study area with sheep having more important role than reported in other areas in Sudan (Omer et al. 2010).

51% of the dogs examined necropsied in Central Sudan were harbouring Echiococcus worms (Saad and Magzoub 1986). In a recent survey including genetic identification (Omer et al. 2011), *Echinococcus* worms were found 53% (35/66) and 44% (8/18) Tamboul and Rofaa cities in central Sudan. Echinococcus canadensis was found as an overwhelming strain and Echinococcus sensu sticto G1 was detected for the first time in Sudan in that study. In the current study, we used zinc chloride flotation to recover taeniid eggs followed by a previously reported nad1RFLP- PCR (Hüttner et al.2009) to investigate both the presence and genetic identification of Echinococcus spp. in dogs and some other carnivores in White Nile area in Sudan. This method is used for the first time in such a survey in Sudan and Africa. It is known that Tatniid eggs in the feces of carnivores are microscopically indistinguishable. Due to this diagnostic uncertainty, this method can be perfectly applied in future survey as it allows identification of eggs to species level.

Taeniiade eggs were detected in 73 stray dogs (48.6%), 3 foxes (60%) and 1 Wild dog (33%). Average number of eggs/1gm faecal sample/animal was 3.2, 4.4 and 1.2 in stray dogs, foxes and wild dogs respectively. Genetic identification confirmed the presence of E. canadensis in 51 dogs (34%) and 1 fox (20%). This result show that *E.canadensis* is prevalent to a high extent in stray dogs in the White Nile area and that foxes (*Vulpes pallida*) can be considered as definitive host for the parasite in the area. Even though, amplification products were not detected form any of the material obtained from wild dogs, future survey including more samples can be included in future surveys. Role of foxes should also be clarified. Moreover, there is a need for a well- articulated control programme which should involve regular anthelmintic medication, as well as incorporation of health education programs

Acknowledgements
The authors gratefully acknowledge financial support by the Alexander von Humboldt Foundation and by the Deutsche Forschungsgemeinschaft (DFG), grant RO 3753/1-1.

References

Benito A, Carmena D, Joseph L, Martinez J, Guisantes JA (**2006)** Dog echinococcosis in northern Spain: comparison of coproantigen and serum antibody assays with coprological exam. Vet Parasitol 142:102-111

Dalimi A, Motamedi G, Hosseini M, Mohammadian B, Malaki H, Ghamari Z, Ghaffari FF **(2002)** Echinococcosis/hydatidosis in western Iran. Vet Parasitol 105:161-171

Eckert J, Deplazes P **(2004)** Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. Clin Microbiol Rev 17:107-135

Gemmell MA, Lawson JR, Roberts MG, Griffin JF **(1990)** Population dynamics in echinococcosis and cysticercosis: regulation of Taenia hydatigena and T. ovis in lambs through passively transferred immunity. Parasitology 101 Pt 1:145-151

Huttner M, Nakao M, Wassermann T, Siefert L, Boomker JD, Dinkel A, Sako Y, Mackenstedt U, Romig T, Ito A (**2008)** Genetic characterization and phylogenetic position of Echinococcus felidis (Cestoda: Taeniidae) from the African lion. Int J Parasitol 38:861-868

Huttner M, Siefert L, Mackenstedt U, Romig T **(2009b)** A survey of Echinococcus species in wild carnivores and livestock in East Africa. Int J Parasitol 39:1269-1276

Huttner M, Siefert L, Mackenstedt U, Romig T **(2009a)** A survey of Echinococcus species in wild carnivores and livestock in East Africa. Int J Parasitol 39:1269-1276

Macpherson CN, Karstad L, Stevenson P, Arundel JH **(1983)** Hydatid disease in the Turkana District of Kenya. III. The significance of wild animals in the transmission of Echinococcus granulosus, with particular reference to Turkana and Masailand in Kenya. Ann Trop Med Parasitol 77:61-73

Macpherson CN, Wachira TM, Zeyhle E, Romig T, Macpherson C **(1986)** Hydatid disease: research and control in Turkana, IV. The pilot control programme. Trans R Soc Trop Med Hyg 80:196-200

Magambo JK, Hall C, Zeyle E, Wachira TM **(1996)** Prevalence of human hydatid disease in southern Sudan. Afr J Health Sci 3:154-156

Magambo JK, Zeyhle E, Wachira T **(1998)** Hydatid disease in Toposaland, Southern Sudan. Afr J Health Sci 5:129-132

Mathis A, Deplazes P, Eckert J (**1996)** An improved test system for PCR-based specific detection of Echinococcus multilocularis eggs. J Helminthol 70:219-222

Muller-Graf CD **(1995)** A coprological survey of intestinal parasites of wild lions (Panthera leo) in the Serengeti and the Ngorongoro Crater, Tanzania, east Africa. J Parasitol 81:812-814

Nakao M, Sako Y, Ito A **(2003)** Isolation of polymorphic microsatellite loci from the tapeworm Echinococcus multilocularis. Infect Genet Evol 3:159-163

NELSON GS, RAUSCH RL **(1963)** ECHINOCOCCUS INFECTIONS IN MAN AND ANIMALS IN KENYA. Ann Trop Med Parasitol 57:136-149

Omer RA, Dinkel A, Romig T, Mackenstedt U, Elnahas AA, Aradaib IE, Ahmed ME, Elmalik KH, Adam A **(2010b)** A molecular survey of cystic echinococcosis in Sudan. Vet Parasitol 169:340-346

Omer RA, Dinkel A, Romig T, Mackenstedt U, Elnahas AA, Aradaib IE, Ahmed ME, Elmalik KH, Adam A **(2010a)** A molecular survey of cystic echinococcosis in Sudan. Vet Parasitol 169:340-346

Rishi AK, McManus DP **(1987)** Genomic cloning of human Echinococcus granulosus DNA: isolation of recombinant plasmids and their use as genetic markers in strain characterization. Parasitology 94 (Pt 2):369-383

Saad MB, Magzoub M **(1986)** Echinococcus granulosus infection in dogs in Tambool, Sudan. J Helminthol 60:299-300

Thompson RC, Kumaratilake LM, Eckert J **(1984)** Observations on Echinococcus granulosus of cattle origin in Switzerland. Int J Parasitol 14:283-291

Troncy P, Graber M **(1969)** [Echinococcosis-hydatidosis in Central Africa. II. Human echinococcosis in Chad]. Rev Elev Med Vet Pays Trop 22:69-74

Verster A, Collins M **(1966)** The incidence of hydatidosis in the Republic of South Africa. Onderstepoort J Vet Res 33:49-72