Journal of Agricultural and Veterinary Sciences

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Echinococcosis: Epidemiology and Genotyping of Echinococcus Speices in Sudan

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Article history: Received: 1 June 2013
Accepted: 22 June 2013

Abstract: Cystic echinococcosis (CE) is considered as a re-emerging disease in various regions, e.g. the Middle East, central Asia, and northern and eastern Africa. In sub-Saharan Africa, CE is highly endemic. In this study, data were collected in abattoir-based surveys in Tamboul town (Central-Eastern Sudan,) and in Nyala abattoir (Darfour region). Out of 1012 camels examined in Tampoul (713) and Nyala (299) an infection rate of 16.1% and 29.1% was reported, respectively. The favorite site for camel's cysts is the lung (81%). Fertility rate of cysts encountered from camels is about 57%. This finding appears to reflect the importance of the camel as a major intermediate host of this zoonotic disease in Tamboul and Nyala area. *Echinococcus* isolates (81) collected from camels were genotyped by PCR-RFLP and specific G5/6/7 PCR. In all cases, the G6 genotype of *E. canadensis* was found. The public-health impact of these finding are discussed in terms of the various species and genotypes of *Echinococcus* and the role of each in human health.
Keywords: Cystic echinococcosis of camel, Prevalence, Molecular genotyping, Sudan
Sequences were submitted to gene bank database, accession No**:** AB271912

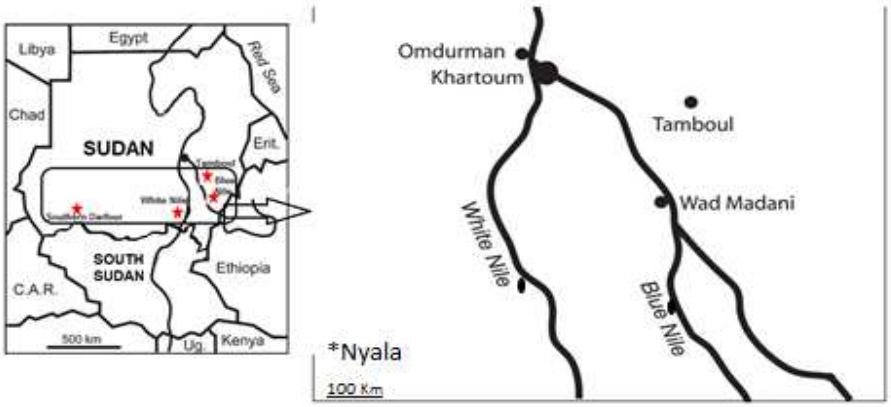
Introduction

Echinococcosis is a disease caused by the adults and larval (metacestode) stages of taeniid cestodes in the genus *Echinococcus*. Four species are recognized in this genus viz *E. granulosus, E.multilocularis, E. oligarthrus* and *E. vogeli*. *Echinococcus granulosus* has a cosmopolitan distribution and occurs in almost all ecological zones (Schantz *et al.*, 1995). The other three species are prevailing in cool climates of the northern hemisphere

and South–Central America. Transmission of *E. granulosus* occurs predominantly in synanthropic cycles where dogs act as the definitive host of *E. granulosus*. The worm shows high fertility in sheep and is mainly transmitted in asheep–dog cycle (Eckert *et al.*, 2001). Many livestock-rearing areas of northern and eastern Africa have high prevalence of echinococcosis, both in the human populations and farm animals (Macpherson *etal* 1997, Eckert *et al.*, (2001). In cenral part of Sudan, ruminants and dogs are frequently affected (El-Khawad *et al.*, 1979, Saad *et al.*, 1986). The traditional methods of animal husbandry adpoted in this area, the homeslaughtering of livestock, the large numbers of stray dogs, and the absence of anthelmintic care for canines all probably favour the transmission of *Echinococcus*. A number of distinct 'strains' of *E. granulosu* shave been described in the past, which exhibits a considerable diversity of morphology, development and host range. This diversity was confirmed by genetic studies which lead to recognition of a minimum of 10 strain-types G1–G10 (Thompson *et al.*, 1995, Lavikainen *et al.*, 2003). In the present study, attempts were made to assess the epidemiological situation of cystic echinococcosis in camels with regards to genotyping of *Echinococcus granulosus*.

Materials and Methods

Study area Surveys were done in Tamboul town (Central Eastern, Sudan), and Nyala area (western Sudan) (Figure 1).



Study area: Southern Darfour state (Nyala), Gezira state (Tamboul)

Abattoir Survey

A total of 1013 camel (713 from Tampoul and 299 from Nyala) carcass were examined in abattoirs, during routine meat inspection, for presence of cystic echinococcosis. Aging of each individual animal was made before examination for cystic echinococcosis.

The liver, lungs, spleen, heart and kidneys from each carcass were carefully examined. Cysts when found were taken to a laboratory, where their diameters and fertility were recorded.

A cyst was considered fertile if the cyst fluid aspirated from it was found to contain apparently viable protoscolices. Samples of protoscoleces or hydatid materials were preserved in 70% ethanol and stored at room temperature for genetic characterization.

Molecular genotyping

DNA extraction

Single protoscoleces were separated using a capillary pipette and lysed in 10 ll 0.02 M NaOH at 95 _C for 10 min (Nakao *et al.*, 2003). DNA was also obtained and the solution was used directly as a template DNA in the PCR.

PCR of the nad1 gene

This method is based on the amplification of a 1073–1078 bp-long fragment including the complete NADH dehydrogenase subunit 1 (nad1) gene. Thermal reactions were performed according to Hüttner et al (2008, 2009). PCR was performed in a 50 ll vol. containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 lM of each dNTP,12.5 pmol Forward primer nadA: 5-TGTTTTTGAGATCAGTTCGGTGTG-3 and Reverse primer nadC: 5-CATAATCAAACGGAGTACGATTAG -3, 1.25 U Ampli-Taq Polymerase (Applied Biosystems) and 5 ll of the total DNA. The amplification conditions for 35 cycles were denaturation for 30 s at 94C, annealing for 30 s at 55 C and elongation for 60 s at 72 C. PCR products were separated on a 3% ethidium bromide-stained agarose gel.

RFLP-PCR of the nad1 gene

PCR product of the nad1 gene was digested by restriction enzyme HphI (Hüttner et al 2008). PCR products were digested for 6 h with the restriction enzyme HphI according to the manufacturer's instruction (Fermentas). Restriction fragments were visualized by gel electrophoreses through a 3% ethidium bromide-stained agarose gel.

PCR specific for *E.ortleppi* and *E.granulosus* G6/7

For the first PCR (G5/6/7) which amplifies 254bp fragment of E. ortleppi (G5) and E. granulosusG6/7,the primer pair E.g.cs1for.(5^{0} ATTTTTAAAATG TTCGTCCTG3^{0}) and E.g.cs1rev.(5^{0}CTAAATAAT ATCATATTACAAC3^{0}) was used.The100µl reaction mixture consisted of 10mM Tris–HCl (pH8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP, 50 pmol of each primer and 2.5units Ampli-Taq Polymerase (Perkin ElmerBiosystems) for 40cycles (denaturation for 30 sec 94°C,annealing for 1min at 53°C and elongation for 40 sec at 72°C (Dinkle, 2004).

The system for diagnosis of *Echinococcus ganulosus* G6/7 and *Echinococcus ortleppi is shown* in Fig 2.To discriminate between *E.ortleppi* and *E.granulosus* G6/7, semi-nested PCRs specific for G6/7(g6/7PCR; primer pair E.g. camel for 5^{0} ATGGTCCACCTATTA TTTCA3^{0} and E.g.cs1rev.) and for E.ortleppi (g5 PCR; primer pair E.g.cattle.for.5^{0} ATGGTCCACCTATTA TTTTG3^{0} and E.g.cs1rev.) were used in a second step,each amplifying adifferent fragment of 171bp. The reaction mixtures of 50 µl contained 1.5 µl of amplification product, 10 mMTris–HCl(pH8.3),50 mMKCl, 2 mM $MgCl_2$ 200 µM of each dNTP, 25 pmol of each primer and 1.25 units Ampli-Taq Polymerase (PerkinElmerBiosystems) for 30cycles (denaturation for 30 sec 94°C,annealing for 1min at 60°C and elongation for 30 sec at 72°C).

Amplification products were resolved on a1.5%ethidium bromide stained agarose gel.

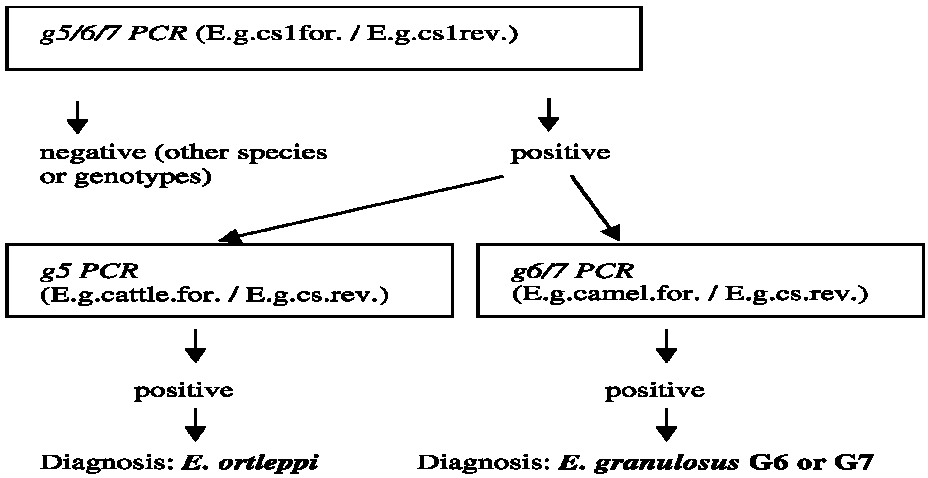


Fig.2 PCR system for diagnosis of *Echinococcus ganulosus* G6/7 and *Echinococcus ortleppi* (G5) (Dinkle, 2004)

Data analysis

Epidemiological data were analyzed by SPSS. For genotyping bands pattern obtained were compared with published band pattern reported by Huettner *et al.,* (2009) for RFLP-PCR and by Dinkle *et al.,* (2004) for specific G5/6/7 PCR (specific G5 PCR and specific G6/7 PCR.

Results

The infection rate of cystic echincoccosis in camels is 16.1% and 29.1% in Tamboul and Nyala, respectively (Table1and Table 2). Camels usually harbor more than one cyst per infected animal. Fertility rate of the cysts encountered from camel is 53%. The lungs are a main predilection site of camel cysts. About 90% of camels slaughtered at Tamboul abattoir were females with age between 4 to 12 years (Table 1). Out of 106 camels with age less than three years only one animal was found to be infected with cystic echinococcosis and the cyst encountered was found to be sterile (Table 1). About 50% of camel cysts were 2-5 cm in diameter and only 24.3% were 5-10 cm diameter (Table 1). Age of camels examined at Nyala abattoir was not reported. 40% of camel cysts were 2-5 cm in diameter and only 16% were 5-10 cm diameter (Table 2)

Amplification of nad 1 gene revealed similar band pattern of 1073-1078 bp-long fragments (Fig 3) a subsequent digestion with restriction enzyme Hph1 confirmed similar banding pattern for G6 (Fig. 4).

A specific and sensitive PCR/semi- nested PCR system for the rapid diagnosis of *Echinococcus granulosus* genotype G6/7,and *Echinococcus ortleppi* (G5) described by Dinkle et al (2004) is proved to be very useful for discrimination of Echinoccus genotypes. The genotype 5/6/7 PCR was found to amplify *E. ortleppi* and G6/G7 genotypes of *E.granulosus* with a characteristic band of 254bp (Fig 5). Using specific G5/6/7 PCR revealed a band of 254 bp (Fig 5).To discriminate between *E. granulosus* G6/7 and *E.ortleppi*, the amplification product under went different semi-nested PCRs (G5 PCR and G6/7 PCR). The G6/7 PCR amplified only the *E.granulosus* G6/7 genotypes (Fig 6), whereas the G5 PCR selectively amplified *E.ortleppi* (Fig.7). Both PCRs resulted in a specific product of 171bp. With this system, no discrimination was achieved between *E.granulosus* genotypes G6 and G7. All isolates genotyped were clearly demonstrate similar band pattern for G6 genotype (RFLP-PCR) or G6/7 genotype using specific G5/6/7 PCR

Table 1: Predilection site, size and biological condition of cysts examined at infected camels at Tamboul abattoir during Dec 2009-Dec 2010

	Recovered from				Diameter			Condition		
Age y/No	Any location	liver	Lung	Other	≤2	2-5	6-10	Fertile	Sterile	Calcified
1-3 (106)	1	-	-	-	1	-	-	-	1	-
4-8 (339)	57	12	45	-	21	25	11	31	19	7
9-12(229)	49	7	43	-	19	16	14	31	12	6
13- (39)	8	2	6	-	-	5	3	4	2	2
Total (674)	115	21	94	-	4	46	28	66	34	15

Table 2: Predilection site, size and biological condition of 129 cysts examined from camel from Nyala abattoir during Dec 2009-Dec 2010

Recovered from		diameter (cm)				Condition		
organ	no. cyst	≥2	2-5	6-10	≤10	Fertile	Sterile	calcified
Lung	348	78	82	58	130	87	12	249
Liver	20	7	06	02	05	06	7	7
Spleen	0 1	0	0	0	01	01	0	0
Total	369	85	88	60	136	94	19	256

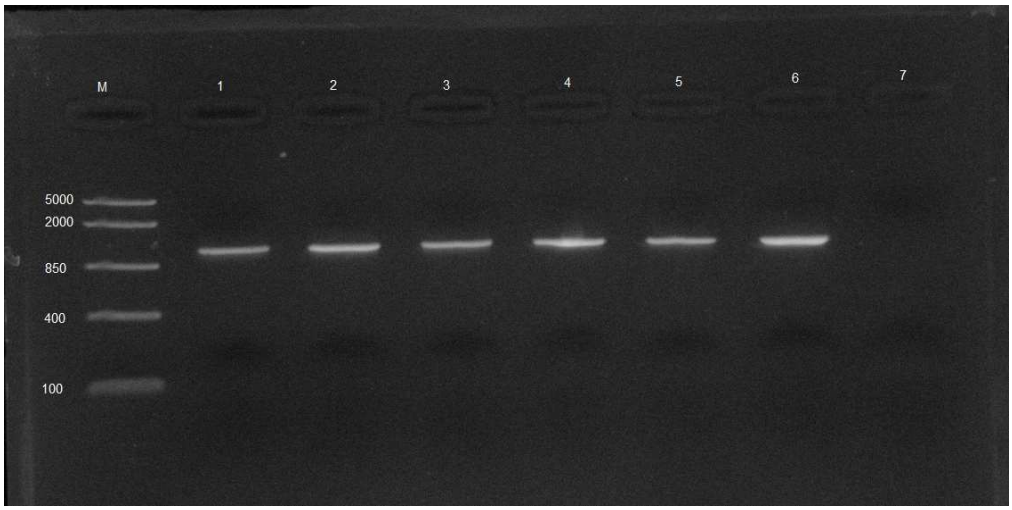


Figure 3: PCR of nad1 gene.
M ; Marker .1-2 ; G5 . 3-4 ; G6 . 5 ; G6 positive control . 6 ; G5 positive control . 7 ; negative control

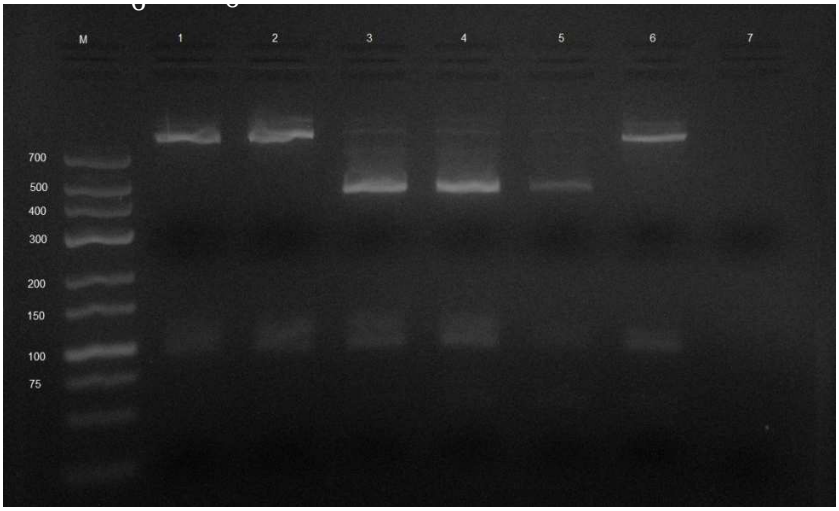


Figure 4: .RFLP-PCR of the nad1 gene with the Hph1.
M ; Marker .1-2 ; G5 . 3-4 ; G6 . 5 ; G6 positive control . 6 ; G5 positive control . 7 ; negative control .

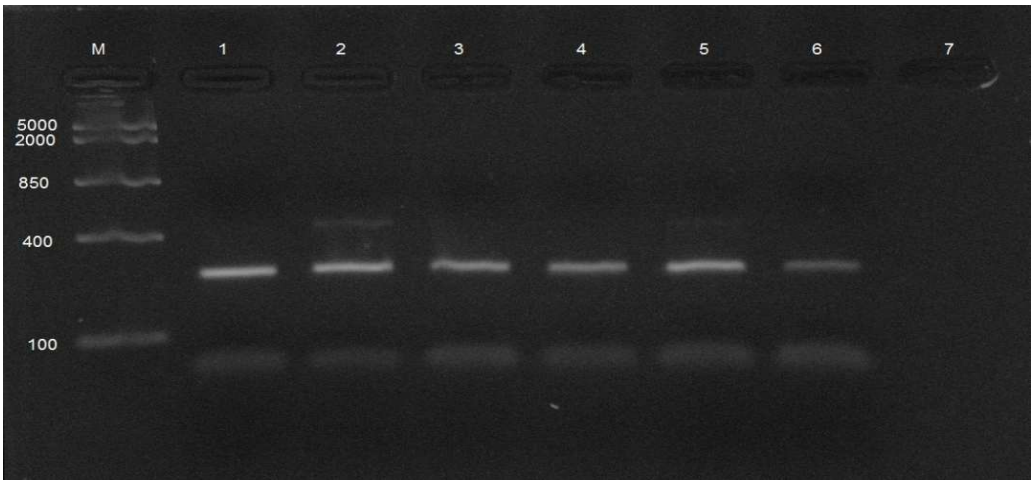


Figure 5:.G5/6/7 PCR, M, marker, 1-2 cattle protoscolece, 3-4 camel protoscolece5 G6 positive control, 6 G5 positive control, 7 negative control.

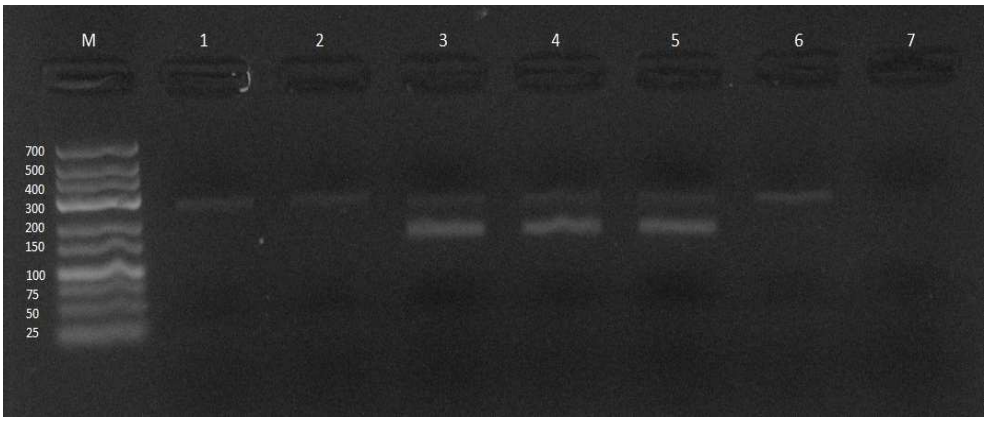


Figure 6.G 6/7 specific PCR, M, marker, 1-2 cattle protoscolece, 3-4 camel protoscolece 5 G6 positive ontrol, 6 G5 positive control, 7 negative control.

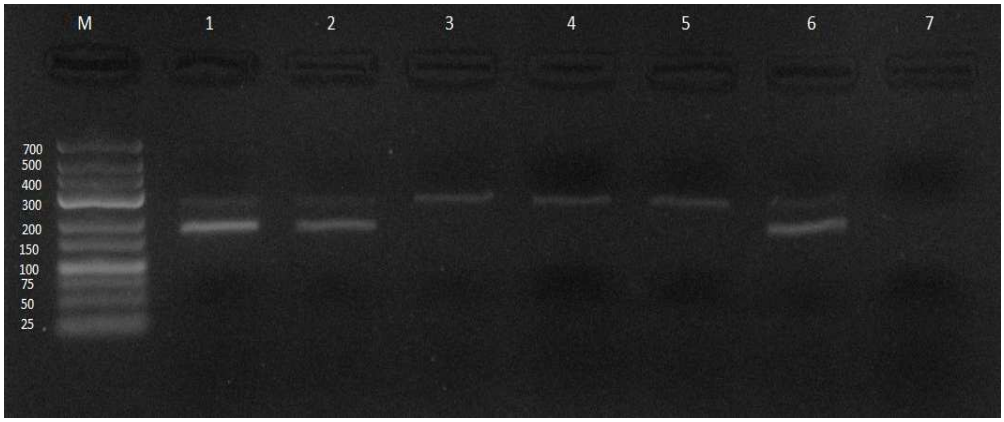


Figure 7: 5.G5 specific PCR, M, marker, 1-2 cattle protoscolece, 3-4 camel protoscolece 3G6 positive control, 6 G5 positive control, 7 negative control.

Discussion

In Sudan several studies documented the endemicity of cystic Echinococcosis in different part of the country (Saad and Magzoub 1989; Elmahdi *et al*; 2004; Osman *et al*., 2007). The result from this survey is lower than those observed, in the same region, by Elmahdi *et al., (*2004); they reported prevalence of 44.6%, 6.9% and 3.0% in camel, sheep and cattle, respectively.

The infection rate reported (16.1%) is likely reflect the true epidemiological situation in camel since only one camel infected in age group less than three years. However, the prevalence of *E. granulosus* is known to be positively correlated with age (Lahmar *et al.,* 1999, Dueger *et al.,* 2001).

Given that 53% of the cysts in camel were fertile, most transmission in Tamboul and Nyala areas seems to be based on camels. This is in contrast to the situation in many other regions of Africa, including parts of southern Sudan, Kenya and the countries of the Moroco, where only sheep are involved in the transmission of *Echinococcus* (Macpherson *et al*., 1997).

In most areas, where CE is a major public health problem, the G1 genotype (which is highly fertile in sheep) predominates. In this study all 81 Echinococcus isolates collected from camel genotyped by RFLP-PCR and specific G5/6/7 PCR were found to be G6 genotype of *E. Canadensis*. Dinkel *et al.,* (2004) previously detected G6 (camel strain) in 44 of the 46 Echinococcus isolates (35 from camels, eight from cattle and three from sheep) collected from the same area.. If the only thing keeping the incidence of human CE in central Sudan low is the rarity of G1 (it is not the rarity of dogs and sheep, both of which are common), then the accidental introduction of this apparently malignant strain from elsewhere has to be prevented. Human infections with genotype G6 ('camel strain') have also been reported (Dinkel *et al.,* 2004). Fifty dogs (33 male, 17 female) were examined for presence of *E. granulosus* worm using sedimentation and counting technique. An infection rate of 76% was reported in dogs (data not shown). It is probably not valid to compare this level of prevalence with that reported for dogs from other regions, as the prevalence in dogs are known to differ significantly on small spatial scales, and are heavily dependent on particular risk behaviors of dogs (such as access to offal) which can differ markedly according to area and season (Wachira *et al*., 1994). Most isolates from humans were previously characterised as *E.granulosus* G1 (common sheep strain) and only few human infections with *E. ortleppi* and *E. granulosus* G6 (camel strain) have been recorded.

Specific PCR for identification of G6/7 and G5 described previously by Dinkle is proved to be useful for epidemiological studies (Dinkle *et al*., 2004). As indicated in this study, 81 *Echinococcus granulosus* isolates genotyped by both RFLP-PCR and specific G5/6/7 PCR were identified as G6 genotypes. However, in most areas where CE is a major public health problem, it is the G1 genotype (which is highly fertile in sheep) that predominates. Dinkel *et al.,* (2004) previously detected G6 (camel strain) in 44 of the 46 Echinococcus isolates (35 from camels, eight from cattle and three from sheep) collected from the same area.

It was recorded from this study that G1 genotype, which is highly infective to human was not reported in this study. However, relative rarity of human CE in Sudan may result from the absence or rarity of G1 genotype. Human infections with genotype G6 (camel strain) have also been reported. Elmahdi (2012) had genotyped Echinococcus isolates from different areas which confirmed the predominance of G6 genotypes with only two isolates reported as G5 genotypes. G1 genotypes were reported by omer (2010) in few isolates from human from Nuba Moutain in bordering Southern Sudan state. G6

genotypes is also reported by Elmahdi, (2006 dogs. The camel strain (G6) of *E. granulosus* was detected for the first time in a human patient from eastern Africa. This strain appears to have limited pathogenicity to man with few previous records from humans. molecular investigations of human cystic hydatid cases from Argentina (Rozenzvit *et al*., 1999) had shown for the first time an involvement of G6, and in Nepal two human isolates have been identified as G6 (Zhang *et al*.,2000). Additionally a study carried out in Mauritania had also identified this genotype from two samples of human origin based on mitochondrial cox1 and nad1gene sequencing (Bardonnet *et al*., 2001). In Kenya also a single human infection with G6 contrasts with 177 human cases of G1 infection, despite the fact that G6 is wide spread and frequent in animal hosts. The identification of Echinococcus infections in dogs and other final hosts is of high priority in epidemiological studies.

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الاكياس العدارية في الابل: الوبائية و تحديد الانماط الجينية لانواع الاكياس العدارية في السودان

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7. قسم الطفيليات–جامعة هونهايم–المانيا الاتحادية

المستخلص

يعد مرض المحبة الشوكية من الامراض التى عاودت الانتشار فى مناطق مختلفة من العالم علي سبيل المثال الشرق الاوسط، اواسط اسيا، و شمال و شرق افريقيا. ويعتبر المرض متوطنا فى افريقيا جنوب الصحراء. اوضحت الدراسة ان الرئة هى المنطقة المفضلة للأكياس فى الابل(81%),معدل خصوبة الأكياس التي تم جمعها من الابل بلغت حوالي (57%).هذه النتائج يمكن أن تعكس أهمية الابل كعائل وسيط رئيس لهذا المرض الحيواني المنشأ في منطقة تمبول و نيالا.تم جمع 81 عترة من طفيل المحبة الشوكية من الابل و تم تحديد نوع الطفيل عن طريق تفاعل البلمرة المتسلسل و تقانة الانزيمات القاطعة و تفاعل البلمرة المتسلسل للعترات لتحديد الانماط الجينية. في كل العينات تم تحديد العترة G6 للمشوكة (*E. canadensis*). نوقشت اهمية نتائج الدراسة للصحة العامة اعتمادا على عترات الطفيل واهميتها لصحة الانسان.