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COMPARATIVE CHARACTERIZATION AND DNA ANALYSIS OF HYDATID AND CYSTICERCUS TAENUICOLLIS CYST FLUIDS

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SUMMARY

Molecular technology is providing highly improved methods for identification of parasites, either of medical, veterinary, biological importance or interest. Hydatid fluid of sheep (HFS), camel (HFC), equine (HFE) and pig (HFP) origin and also *Cysticercus taenuicollis* fluid of sheep (CTFS) and pig (CTFP) origin were used in this investigation. Electrophoretic profile of metacestode fluid antigens using SDS-PAGE revealed multiple components in both high and low molecular weight ranges. There was extensive electrophoretic similarity between the investigated fluid antigens especially at 83 KDa and a common band for all antigens except CTFP at 243 KDa. Extensive cross-reactivity between metacestode fluid antigens in EITB using hyper-immune serum of hydatid cyst fluid of camel origin. There was common polypeptide band at 83 KDa recognized among all species. Cross-reactive components between all antigens except CTFS recognized by antiserum of HFC at 116 KDa and also between all antigens except HFS at 95 KDa. Further investigations will be necessary to isolate the cross-reactive antigens and to evaluate their potency in protection against heterologous infection. The amplified restriction fragment length polymorphism (AFLP) technique is a relatively new method for the analysis of polymorphism that has not yet been widely used in parasitology. In this article, DNA analysis gave similar fragment for HFS, HFE, HFC, HFP, CTFS and CTFP at 293 Kpb and a common fragment at 305 Kpb in *C. taenuicollis* and hydatid fluids expect camel origin. These findings may have important consequences for human health and the control of hydatid and cysticercosis diseases.

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INTRODUCTION

Hydatidosis caused by the metacestode of dog tapeworm *Echinococcus spp.*, it is a global zoonotic infection, which is economically important and constitutes a threat to public health in many countries. Transmission is most intense in livestock raising regions where veterinary services are unsatisfactory and where offal from slaughtered animals is accessible to dogs. It is a wide spread infection through the world and it occurs in all domestic live stock (Eckert et al., 2001). In Egypt, a survey on the frequency of hydatid disease in slaughtered animals (Derbala and Zayed 1997) revealed that the percentage in camels, donkeys, pigs, sheep, buffaloes and cows are 40, 7-69, 0.90, 0.77, 0.0 and 0.0%, respectively. Moreover, it was found that, the fertility in hydatid from camel origin is higher than that from other origins. El-Ridi et. al., (1990) estimated the prevalence of Echinococcus antibodies in camels using the indirect haemagglutination test (IHAT) and found that out of 100 camels, 27 camels were seropositive. The infection pressure to camels was somewhat lower in comparison to sheep reported in an earlier study (Lahmar et al., 2004). However, because camels are much longer-lived animals, the results of the model fit suggested that older camels have a relatively high prevalence rate, reaching a most likely value of 32% at age 15 years. This could represent an important source of transmission to dogs and hence indirectly to man. There was no evidence of parasite-induced immunity in camels (Lahmar et al., 2004). *Cysticercus taenuicollis*, is a metacestode of canine tapeworm *Taenia hydatigena*. It has been commonly reported in the liver, mesentery and omentum of cattle, sheep, goats, pigs, squirrel and other wild ruminants (Jones and Hunt, 1983). The pathology induced by the migrating cyst has been scantly studied (Darzi et al., 2002), misdiagnosis of hydatid cysts by ultrasound scaning alone can occure (Hira et al., 1988). It's characterized in the late symptomatic stage when significant pathology has already occurred. (Pawlowski, 1997). Immuno-diagnosis is highly efficient and may provide specific confirmation of CE (Craig, 1997). Leaking cyst fluid antigens are known to induce high levels of specific parasite antibodies in patients sera (Leggatt et al., 1992). While Bandyopadhyay and Singh (2000) revealed that the hydatid cyst fluid is the most widely used antigen for serological diagnosis of animal hydatidosis. Individual cyst fluid proteins (Shepherd and McManus, 1987) and cloned cyst fluid polypeptides (Shepherd et al., 1991) were used as possible diagnostic molecules. Enzyme linked inmunoelectrotransfer blot (EITB) was reported to be the most sensitive serological assay for confirmation of hydatidosis (Omar et al., 2003). Also Ramadan et al., (1999) and Kandil et al., (2003) reported that the specificity of EITB test was 100%. Further more, using the molecular techniques for DNA analysis resulted identification of nine distinct genotypes (G1- G9) of *E. granulosus* (Bowles and McManus 1993 a, b; Le et al., 2002).

The random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) detected DNA poly-morphism. The technique involved the random recognition and amplification of a limited number of loci randomly distributed throughout the genome due to their hybridization with the primer used. The obtained PCR products produce a banding pattern that is revealed by agarose gel electrophoresis. The RAPD-PCR is technically non-radioactive, rapid, simple, highly sensitive, requiring only small amount of DNA (routinely 25 ng) for analysis and it does not require previous sequence knowledge for the synthesis of suitable primers (Azab et al., 2004). Amplified fragment length polymorphism (AFLP) is a whole genome finger printing method based on random amplification of the DNA in order to determine the degree of variation polymorphism between *E. granulosus* cysts collected from different animal species (Verastegui et al., 2003; Yamasaki et al., 2004).

The aim of this investigation was planed in order to study the antigenic and genomic variations of metacestode fluids collected from different animal origins, which would be useful for diagnosis and vaccine development strategy.

MATERIALS AND METHODS

1- Collection of hydatid and *Cysticercus taenuicollis* cyst fluids: Hydatid and *Cysticercus taenuicollis* (CT) cysts from lung, liver and omentum of infected sheep, camel and pigs were obtained from El- Bassatein abattoir, Cairo, Egypt. While hydatid cysts of equine origin were obtained from slaughtered donkeys in the Zoo, Giza, Egypt. Hydatid fluids were collected from each fresh cyst and concentrated in a dialysis bag against polyethylene glycol (8000 MW Sigma Co, USA). Protease inhibitors, 2mM (10 mg/ 100 ml PBS-PH 7.2) were added overnight at 4°C. Protein content of the obtained hydatid fluid (HF) and *Cysticercus taenuicollis* fluid (CTF) was measured according to Bradford (1976).

2-Antigens preparation: Fluids of hydatid and CT cysts were aspirated aseptically and checked for the presence of protoscoleces. The fluids were filtered using a Seitz filter (Carlson Ford Sales, Lid., Ashton-under-Lyne. United kingdom) and checked for the sterility aerobically. The fluids were centrifuged at 10,000 g. for 30 min, the supernatant fluid obtained from each animal origin was considered as a crude antigen (Devt and Parija 2003).

Hydatid fluid of sheep origin	: HFS
Hydatid fluid of camel origin	: HFC
Hydatid fluid of equine origin	: HFE
Hydatid fluid of pig origgin	: HFP
C.taenuicollis fluid of sheep origgin	: CTFS
C.taenuicollis fluid of pig origgin	: CTFS

3-Hyperimmune sera: The immune sera were prepared in rabbits against the hydatid cyst

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fluid of camel origin according to Azwai et al., (1993).

4- Polyacrylamide gel electrophoresis: The gel cast comprised 12% resolving & 4% stacking gels & applied 100 µl protein/well. Mini-protein II Dual slab cell (Bio-Rad Labs, Richmond, CA) was used to conduct electrophoresis using discontinuous system of Laemmli (1970). Each fluid antigen was mixed in buffer (12% Tris Hcl, pH 6.8 and 0.25% SDS) and bromophenol blue was added as the tracking dye. The gels were stained with 0.1% Coomassie blue R-250 in fixative (25% methanol 10% acetic acid and 65% distilled water) destained for overnight. Analysis of the separated bands was performed by software analysis gel proanalyzer.

5- Enzyme linked immunoelectrotransfer blot (EITB): The immunogenicity of cyst fluid antigens was evaluated by immunoblot. All fluids were separated by SDS-PAGE and then electroblotted on nitrocellulose papers (Towbin et al., 1979) in a blotting system. Nitrocellulose paper was incubated with the diluted rabbit antiserum against HFC. After washing paper was incubated with alkaline phosphatase conjugated anti-rabbit IgG, then exposed to substrate solution for 30 min nitrocellulose sheets rinsed with distilled water to stop the reaction. Molecular weight of the detected polypeptide bands was calculated according to the software analysis gel proanalyzer.

6-Extraction of DNA from fluids:

Extraction of DNA from the collected fluids were conducted according to Sambrook et al., (1989) Fluid samples were incubated for one hr at 37°C with 10 volumes of lysis buffer (10 mM Tris Hcl, 100 mM EDTA and 1% SDS) to which add 100-300 µg of proteinase K (Gibco, life technologies) per one ml sample volume. Each sample mixed well and incubated at 56°C overnight with shaking. The samples then cooled and subjected for phenol-chloroform isoamyle-alcohol extraction, followed by ethanol precipitation by adding double volume absolute ethanol and 0.1V sodium acetate, incubate at 20°C overnight then centrifuged at 14.000 rpm for 30 min followed by addition of 70% ethanol, then dry the pellet and dissolve in 20 µl Tris EDTA buffer, RNAse digestion followed by measuring the DNA quantity at 260 nm.

7-Amplified fragment length polymorphism (AFLP): AFLP was carried out by using 3 random primers, MWG Co: Germany. P_1 ACGCGCATGTC, P_2 GAAGCCAGCCC, P_3 GACCAATGCC. Reaction volume were 50 µl and composed of 100 pg DNA, 20 reaction volume were 50 µl and composed of 100 pg DNA, 20 mM dNTP mix (Gibco, life technologies) 5 µl of 10x PCR buffer containing 1.5 mM $MgCl_2$.and 1µTaq Enzyme [Finsyme Co., Finland]. Cycle of PCR was carried out at 93°C for 1 min, times and proceeded by initial denaturation at 94°C for 3 min and final exten-

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sion for 10 min at 27°C. Ten μ l from each product was electrophoresed at 2% agarose containing 0.5 μg ethidium bromid. (Mayta et al., 2000). Marker was used Ø x DNA marker (Finzyme Linland).

RESULTS

I. Electrophoretic profile of the investigated antigens (Fig. 1 Table 1): The results demonstrated complex separation pattern of antigens under study. HFS showed 5 polypeptides at 243, 83, 44, 40 and 32 KDa. Also, HFC separated into 3 polypeptides at 243, 153, and 83 KDa. The HFE differentiated into 13 polypeptide bands ranged from high molecular weight to low molecular weight at 243 to 15 KDa. The HFP separated into 5 polypeptide bands at 275, 243, 153, 83 and 44 KDa. CTFS separated into 10 Polypeptides from 305 to 11 KDa. While the CTFP showed only 2 polypeptide bands at 215 and 83 KDa. There was extensive electrophoretic similarity between the investigated fluid antigens especially at 83 KDa and there was a common band between all antigens except CTFP at 243 KDa. Also, there was a common band between fluids of CTFS, HFS, HFE and HFP at 44 KDa. In addition to presence of common band between HFC, HFE and HFP at 153 KDa. Only one common band was detected between HFS and HFE at 40 KDa and also there was one common band between HFS, HFE and CTFS at 32 KDa.

II. Identification of antigenic targets recognized by the antiserum of camel origin (Fig. 2 Table 2): These targets were probably significant contributors to detect specific protein antigens for the antisera against HFS, HFP, HFE, CTFS and CTFP. Identified seven bands 134, 124, 116, 108, 83, 70 and 52 KDa in the HFS. HFC recognized eight polypeptides 134, 124, 116, 108, 95, 87, 83 and 57 KDa. While HFE detected three polypeptide bands at 116, 95 and 83 KDa. Five bands were recognized in HFP at 134, 124, 116, 95 and 83 KDa. The CTFS separated into 3 bands at 124, 95 and 83 KDa. The CTFP separated into four polypeptides, these were 116, 108, 95 and 83 KDa. Common polypeptide band at 83 KDa was recognized among all species. Also common reacted polypeptide band was recorded at 134 KDa in HFS, HFC and HFP. Cross-reactive component in all antigens except CTFS was recognized by antiserum of HFC at 116 KDa. Also there was extensive cross-reactivity between all antigens except HFS at 95 KDa. Furthermore, cross-reactive band found between HFS, HFC, HFP and CTFS at 124 KDa.

III. Complexity of the AFLP finger prints (Fig. 3 Table 3): In this study AFLP was carried out for demonstrating genotyping of hydatidosis and cysticercosis from different hosts. Finger prints of the lowest complexity were these obtained from HFC, HFP and CTFP consisting of approximately four fragments. CTFS showed patterns containing five fragments,

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while HFS, HFE showed pattern containing about seven and eight fragments respectively. DNA analysis gave similar fragment for HFS, HFE, HFC, HFP, CTFS and CTFP at 293 Kpb. While at 305 Kpb common fragment in HFS, HFE, HFP, CTFS and CTFP was present. However, there was a common fragment between HFS, HFE, HFC and CTFP at 1078 Kpb. Identical fragment between HFS, HFE, HFC and CTFS at 563 Kpb was present.

Common fragment was recorded between HFS, HFE and HFP at 240 Kpb, Also common fragment was showed between HFS, HFC and CTFP at 210 Kpb. Moreover a common fragment was present between HFE and HFC at 887 Kpb and a common fragment was recorded between HFP and CTFS at 270 Kpb. In addition to presence of similar fragment between HFS and HFE at 256 Kpb. Also one characteristic fragment was recorded for CTFS at 614 Kpb.

Table (1): Electrophoretic analysis of metacestode fluids drived protiens (kDa) by SDS-PAGE.

M Wt. Standard	Hydatid fluid				*C. taeniusolis* fluid	
	HFS	HFC	HFE	HFP	CTFS	CTFP
					305	
				275	281	
	243	243	243	243	243	
			215		200	215
			179		181	
		153	153	153		
			124			
97						
					113	
	83	83	83	83	83	83
	44					
45			44	44	44	
	40					
			40			
30	32		36			
			32			
			28		32	
			24			
14.3			15			
					11	

HFC: hydatid fluid of camel origgin
HFE: hydatid fluid of equine origin
HFS: hydatid fluid of sheep origin
HFP: hydatid fluid of pig origin
CTFS: *C. taenuicollis* fluuid of sheep origin
CTFP: *C. taenuicollis* fluuid of pig origin

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Table (2): Identification of cross-reactive polypeptide bands recognized by anti-HFC rabbit serum using (EITB).

M Wt. Standard	Hydatid fluid				*C. taeniusolis* fluid	
	HFS	HFC	HFE	HFP	CTFS	CTFP
-	134	134		134		
	.124	124		124	124	
	116	116	116	116		116
106	108	108				108
		95	95	95	95	95
		87				
80	83	83	83	83	83	83
	70					
		57				
49.5	52					
32.5						
27.5						
18.5						

HFC: hydatid fluid of camel origgin

HFE: hydatid fluid of equine origin

HFS: hydatid fluid of sheep origin

HFP: hydatid fluid of pig origin

CTFS: *C. taenuicollis* fluuid of sheep origin

CTFP: *C. taenuicollis* fluuid of pig origin

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Table (3): PCR-amplified fragments by using DNA from metacestode

Marker KbP	Hydatid fluid				*C. taeniusolis* fluid	
	HFS	HFC	HFE	HFP	CTFS	CTFP
1.078	1.078	1.078	1.078			1.078
0.0.893						
		0.887	0.887			
0.872					0.614	
	0.563	0.563	0.563		0.563	
0.603						
0.310						
0.307	0.305	0.305		0.305	0.305	0.305
0.281	0.293	0.293	0.293	0.293	0.293	0.293
0.271				0.270	0.270	
	0.256	0.256				
0.234	0.240	0.240		0.240		
	0.210	0.210				0.210
0.104						
0.072						

HFC: hydatid fluid of camel origgin

HFE: hydatid fluid of equine origin

HFS: hydatid fluid of sheep origin

HFP: hydatid fluid of pig origin

CTFS: *C. taenuicollis* fluuid of sheep origin

CTFP: *C. taenuicollis* fluuid of pig origin

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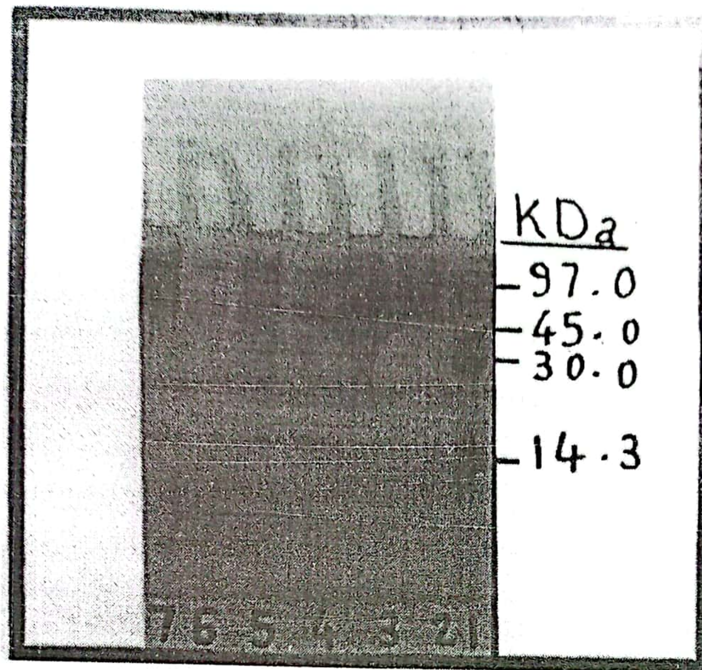

Fig. (1): SDS-PAGE pattern of fluid antigens. Molecular weight standard (lane 1), HFS (lane 2), HFC (lane 3), HFE (lane 4), HFP (lane 5), CTS (lane 6) and CTP (lane 7).

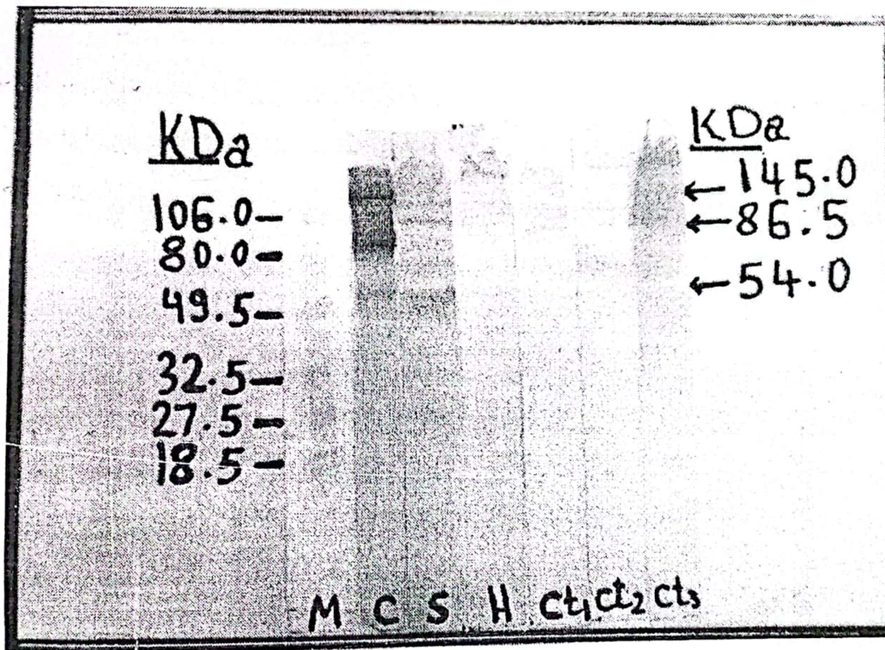

Fig. (2): Identification of cross-reactive polypeptide bands recognized by rabbit anti-HFC using (EITB). M: molecular weight standard; 1: HFS; 2: HFC; 3: HFE; 4: HFP; 5: CTS and 6 CTP.

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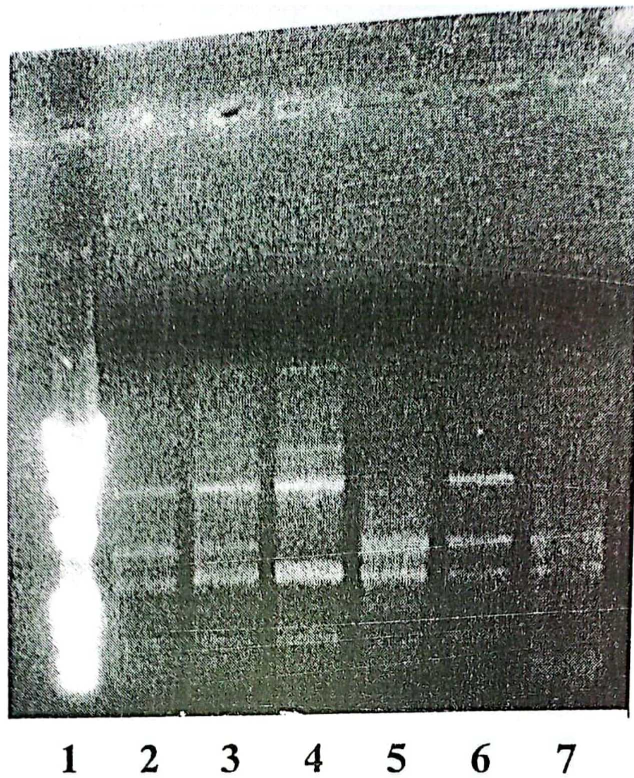

Fig. (3): PCR-amplified fragments by using DNA from metacestode fluids. Hind III digest Marker (lane 1), HFS (lane 2), HFC (lane 3), HFE (lane 4), HFP (lane 5), CTFS (lane 6) and CTFP (lane 7).

DISCUSSION

In the present study, detection of various antigenic fractions from hydatid and *C. taenuicollis* fluid for diagnosis of hydatidosis and cysticercosis in different hosts was carried out. The different strains of *E. granulosus* have been separated on the basis of morphological, developmental and biochemical criteria. Studies have shown that isolates of *E. granulosus* from different hosts have similar in vitro growth characteristics this agreement with Macpherson and Smyth (1985) and Omar et al., (2003). They suggeted that the strains were similar (if not identical) in nutritional and physiological requirements. Additionally, hydatid larval material of sheep, cattle, pig and equine origin slightly differs in morphology, protein characterization and DNA finger prints (Azab et al. 2004). The results showed five specific polypeptide bands in HFS at 243, 83, 44, 40, and 32 KDa and showed three bands at 243, 153 and 83 KDa in HFC. In addition to presence of thirteen band at 243, 215, 179, 153, 124, 83, 44, 40, 36, 32, 28, 24 and 15 KDa in HFE. Moreover, five bands at 275, 243, 153, 83 and 44 were detected in HFP. However, CTFS recognized ten bands at 305, 281, 243, 200, 181, 113, 83, 44, 32 and 11 KDa. While, two bands were detected in CTFP at 215 and 83 KDa. Omar et al., (2003) detected four specific bands in hydatid fluid of sheep origin at 182.1, 132.1, 91.2 and 74.2 KDa. Also they recorded two bands at 179 and 126.2

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KDa in equine origin and non specific protein fraction could be identified in camel origin in Labia. While Itagaki et al. (1994) recognized polypeptide bands at 50, 27 and 14 KDa in the sheep cyst fluid. However, Shambesh et al. (1995) recorded two polypeptide bands at 100 and 130 KDa from hydatid fluid of camel origin and they have the same molecular weight antigens as for hydatid fluid of sheep and cattle origin.

Hydatid fluid of naturally infected sheep, equine, camel and *C. taenuicollis* fluid showed migration for 49.5 and 134 KDa and assayed in immunoblot for reaction with hyperimmune sera against HFC in agreement with Omar et al., (2003) and Itagaki et al., (1994). Shambesh et al., (1995) reported that camel hydatid fluid could be an important source of diagnostic antigen for hydatidosis in the Middle East endemic region. Our results strongly suggest that the common antigenic component of *E. granulosus* and *C. taenuicollis* at approximately 83 KDa. The data suggested that detection of these polypeptide bands could be used in the diagnosis of animal hydatidosis. Trials to detect antigenic polypeptides have been directly carried out by western blotting (Kandil et al., 2003, Omar et al., 2003 and Ito et al., 1993). Gottstein et al., (1991) reported that the main antigenic components of the metacestode at 54-55 and 65-66 KDa but they are not always species specific. Fractionation of the tested hydatid and cysticercus fluid in our study show relatively large numbers of different protein bands of closely related molecular weight. These results agreed with these obtained by Omar et al., (2003), Siles- Lucas and Cuesta-Bandera (1996), where they studied strain differentiation of hydatid cyst species and at the same time reflect antigenic variant between them.

The amplified restriction fragment length polymorphism (AFLP) technique is a relatively new method for the analysis of polymorphism that has not yet been widely used in parasitology in Egypt. Using of molecular techniques for DNA analysis resulted identification of five distinct genotypes (G1-G9) of *E. granulosus* (Bowles and McManus, 1993, a. b, Le et al., 2002). The RAPD-PCR with the 3 primers allowed distinction at the genus level between *E. granulosus* isolated from 4 hosts and other members of the same family (metacestode) the differential amplification may be due to sequence differences in the priming sites (Williams et al., 1990, 1993 and Azab et al., 2004) or due to point mutation, which allow or a bolish primer binding (Hedrick 1992). Hydatid and *C. taenuicollis* fluid of different hosts using the primers under assay, ACGCGCATGTC, GAAGCCAGCCC, and GACCAATGCC, allowed easy distinction between isolates from different hosts. DNA analysis gave similar fragment for HFS, HFE, HFC, HFP, CTFS and CTFP at 0.293 Kpb, while at 0.305 Kpb common fragment in HFS, HFC, HFP,CTFS and CTFP. In addition to high similarity in the pattern between CTF of sheep and

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pig origin at 0.305 and 0.293 Kpb. These results were in agreement with Azab et al., (2004). They found that amplified DNA fragments were common to *E. granulosus* isolates from the level of heterogenity was low in three of human isolates, camel and pig strains. Human and camel isolates were the most related pair, having similar patterns and the highest similarity coefficients. These primers may be considered useful for differentiation at the species or the genus level. The demonstrated difference in the amplification efficiency of different hosts are in agreement with Azab et al., (2004), Hadrys et al., (1992) and Kantanen et al., (1995) they found that the overall level and similarity varied between primers. The primers proved to be useful for distinguishing isolates previously categorized common or different genotypes by mitochondrial DNA sequencing (Bowles et al., 1992). Siles-Lucas et al., (1996) reassessed the genomic identification of a large number of Spanish isolates using five different primers. Reddy et al., (1998) demonstrated significant DNA polymorphism between isolates bubaline strains of bovine. AFLP finger printing was reflected on the values of similarity coefficients calculated between *E. granulosus* isolates from sheep, camel, equine and pig origin. Our results were in agreement with Azab et al., (2004), they found that the similarity coefficient between the camel and pig isolates (82.9%). Also our results agree with the results of Bowles and McManus (1993a), they found close similarities between the camel and pig strains. In a genetic study performed by Rozenzvit et al., (1999) using PCR-AFLP analyzed and DNA sequencing, they demonstrated, for the first time, the presence of G6 genotype (camel strain) in humans in Argentina.

In the present study, some variations within *E. granulosus* isolates from the different host origins were observed. Although, the band patterns were not absolutely invariant within sheep, equine, camel, and pig isolates. The level of heterogeneity, which appeared mainly as differences in band intensities, this is consistent with results of Omar et al., (2004) and Siles-Lucas et al., (1993) who recorded some variability within Spanish ovine, equine and swine strains, with a basic strain specific pattern of RAPD product that allows their identification. Regardless of the primer used. Scott and McManus (1994) recorded some variability within ovine and equine strains from distinct geographical areas. In general there was a great degree of similarity between the different isolates of hydatid and *C. taenuicollis* fluids. These results agree with (McManus and Rishi 1989, Bowles and McManus 1993 a & b, Siles-Lucas et al., 1994), while our results disagree with Omar et al., (2004) and Ortona et al., (1996), who demonstrated some genetic variability within Italian *E. granulosus* sheep strain genotype and found that the amplification patterns of pulmonary isolates were different from those of hepatic isolates.

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In conclusion, the study high lights the usefulness of AFLP finger printing technique in detecting the genetic variability among *E. granulosus* isolates obtained from the different hosts. The use of three primers is very important for the discriminatory power of the technique.

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