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Title:

Molecular characterization of camel, sheep and goat *Echinococcus granulosus* isolates in Riyadh, Saudi Arabia

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Running title: *Echinococcus granulosus* isolated from Saudi Arabian animals

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Abstract

The tapeworm, *Echinococcus granulosus*, causes a major public health as its larval stage causes the life threatening cyclozoonotic disease, in different parts of the world. *E. granulosus* were isolated from different livestock (camel, sheep and goat) of Riyadh in Saudi Arabia. The possibility of intra-genotype variation was investigated using randomly amplified polymorphic DNA (RAPD) analysis by 20 random primers of 8-10 mers. After isolation of protoscolices from fertile cyst, DNA was extracted and Internal Transcribed Spacer gene 1 (*ITS1*) was amplified using specific primer and followed by Restriction Fragment Length Polymorphism (RFLP) with the four restriction enzymes (HhaI, AluI, MspI & RsaI). Our study showed no *ITS1* variants could be detected and no genotypic variation among Saudi animal isolates of *E. granulosus* on the basis of RAPD fingerprinting and RFLP-PCR.

Keywords:

Echinococcus granulosus, Genotype identification, ITS1-PCR, PCR-RFLP, RAPD-PCR and Saudi Arabia.

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INTRODUCTION

Echinococcus granulosus, one of the smallest tapeworms of the Taeniidae family, causes a major public health and veterinary importance as its larval stage causes the life threatening cyclozoonotic disease cystic echinococcosis (CE), in different parts of the world (Eckert & Deplazes, 2004). Many domestic and wild animals act as intermediate hosts harboring hydatid cysts in their liver, lungs and other internal organs. Human can serve as end-stage, accidental hosts, since the life cycle cannot be completed. The adult worms infect wild and domestic canids such as wolves (*Canis lupus*) and domestic dogs (WHO/OIE Manual, 2002). Many studies have indicated that hydatid cysts are commonly found in sheep, camels, cattle and goats in different countries as Iran and Indian (Arbabi et al., 1998; Shareif, 2000; Ahmadi, 2005; Bhattacharya et al., 2008; Rokni, 2009).

In Riyadh, Saudi Arabia hydatid cysts are commonly found in camels, sheep and goats. Ten different genotypes (G1–G10) have been described for *E. granulosus* based on analysis by mitochondrial and nuclear genetic markers (Bowles et al., 1992, 1994; Lavikainen et al., 2003; Saarma et al., 2009; Scott et al., 1997; Thompson, 2008).

Saudi camel, goat, and sheep are considered important hosts in the life cycle of *E. granulosus*, such animals affect on the economic importance of the livestock and human health in Saudi Arabia. To the best of our knowledge, no previous studies have been done to compare the genotypes of *E. granulosus* isolates in the hosts aforesaid.

Various genotypes show differences in morphology, life cycle pattern, transmission dynamics, development rates, host range, pathogenicity and sensitivity to chemotherapeutic agents (Thompson et al., 1995). These differences have important implications in epidemiology and control of hydatid disease. Several molecular studies have identified the presence of two distinct genotypes including the common sheep strain (G1) and camel strain (G6) of *E. granulosus* (Sharbatkhori et al., 2010). However, there are some contradictions in the results. For instance, Harandi *et al.* (2002) could detect G6 genotype in different intermediate hosts including camels, sheep, cattle and humans whereas some surveys failed to detect the G6 genotype; others have found G6 only in camels.

The aim of this study was to evaluate the genetic profile of camel, sheep and goat isolates of *E. granulosus* on the basis of RAPD-PCR and PCR-RFLP to identify ITS1 variant. Variations were investigated among different species of the intermediate hosts and between hosts of the same species.

MATERIALS AND METHODS

Hydatid cysts were collected from liver of camel, sheep and goat during the period of October 2012- June 2013 from slaughter houses in Riyadh city, Saudi Arabia. The Study and all procedures were carried out in accordance with institutional guidelines for human and animal care and use. Only isolates with clean and transparent cyst fluid and the whitish germinal layer were chosen for molecular processes. Protoscolices were aspirated from fertile cysts and rinsed extensively in physiological saline, fixed in 70% ethanol and washed with sterile distilled water to remove the ethanol residuals. Finally, isolates were preserved at −20°C until use for DNA extraction (Table I).

Table.I. Host and number of *Echinococcus* cysts

Samples of hydatid cysts collected from Riyadh KSA from hosts liver.					
Host		Camel, Sheep and Goat			
Number of cysts found in each host		2 or 4			
4		final total			
Serial number	species	Number of samples kept in freezer at -80 °C			
		Tissues	hydatid cysts		Germinal layer
		Eppendorf	Centrifuge tubes		Centrifuge tubes
			scolices	Fluid	
1	camel	16	8	7	2
2	sheep	22	19	10	1
3	goat	8	3	3	1

DNA extraction:

The total genomic DNA from each cyst was extracted using Q-BIO gene kit, USA and its concentration was determined using Nanodrop and stored at -20°C till further use.

Molecular Analysis:

Eighteen *E. granulosus* DNA samples, representative of different intermediate hosts (5 camels, 5 sheep and 5 goats) and 3 of infected liver tissue were examined by RAPD-PCR. Amplification was carried out in a total volume of 25 µl of 12.5 µl master mix of GoTaq® Green Master Mix (Promega, Cat # M712c), 10.5 µl RNAse free water, 1 µl DNA extraction, 1 µl primer (10pmole), RAPD-PCR reaction was run as follows 5 min at 95 °C followed by 45 cycles of 1 min at 94 °C,1 min at 35 °C and 1 min at 72 °C. Finally, an extension step of 10 min at 72 °C was carried out and cooling to 4°C. The amplified DNA products were separated by electrophoresis on 2% agarose gel. The DNA bands stained by ethidium bromide (SIGMA, USA), finally, visualized under UV light (Sambrook and Russel, 2001) and digitally photographed. RAPD band patterns of all 18 samples were compared with each other for each primer. The data were analyzed and the similarity coefficients (S) between isolates were calculated according to Nei and Li (1979).

Genomic DNA samples were examined for *ITS1* gene by PCR using 2 primers, the forward was 5'-GTCGTAACAAGGTTTCCGTA-3', and the reverse was 5' -CTAGATGCGTTCGAA(G/A)TGT CGATG-3'(Invirtogen, Germany). The PCR reaction was carried out as follow: 95 °C for 5 min, 35 cycle of 94 °C for 1 min, 50°C for 1 min and 72 °C for 1 min, and finally an extension step at 72°C for 10 min. PCR-mediated RFLP was carried out according to Bowles and McManus, (1993).

The PCR products were separated by agarose gel electrophoresis (1.5% agarose) and stained with ethidium bromide. The amplicons were extracted from gel using clean Quick gel extraction kit (Qiagen, Cat. No. 28704). 10µl gel PCR purified products derived from *E. granulosus* were digested with 10 units restriction endonucleases *MspI*, *Rsa1*, *AluI* and *HhaI* for 4 h, using buffers recommended by the manufacturer (Promega, USA). Restriction fragments were separated by gel electrophoresis on 2 % agarose.

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RESULTS

In the present work we used RAPD-PCR and RFLP-PCR to characterize *E. granulosus* DNA isolated from cysts of camel, goat and sheep in Saudi Arabia. Twenty primers were used in the RAPD-PCR, in *E. granulosus* isolates. The RAPD-patterns of all camel, sheep, goat isolates were identical. The ITS1-PCR and ITS1-PCR-RFLP with BD1 and 4S primers yielded two amplification products.

Echinococcus granulosus isolates were examined by PCR-RFLP analysis of the ITS1 region from 15 fertile cysts 5 from each of camel, sheep and goat which produced after digestion of the ITS1 fragments using four restriction endonucleases (*MspI, RsaI, HhaI* and *AluI*). The result of RFLP patterns of all camel, sheep and goat isolates were identical (Figs. 1-8).

Fig.1. Ethidium bromide stained (2%) agarose gel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *HhaI*. first comb lanes 2,4,6&8 are undigested camel scolices and lanes1,3,5, &7are digested camel scolices. Second comb lanes 2,4,6,8&10 are undigested sheep scolices and lanes1,3,5,7 &9 are digested sheep scolices.

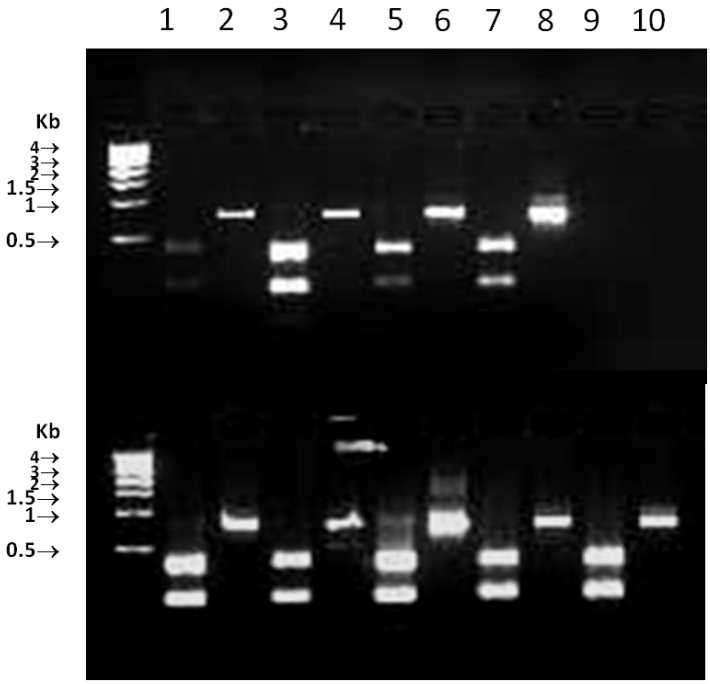

Fig.2. Ethidium bromide stained (2%) agarose gel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *HhaI*. Lanes 2,4,6&8 are undigested goat scolices and lanes1,3,5 &7are digested goat scolices.

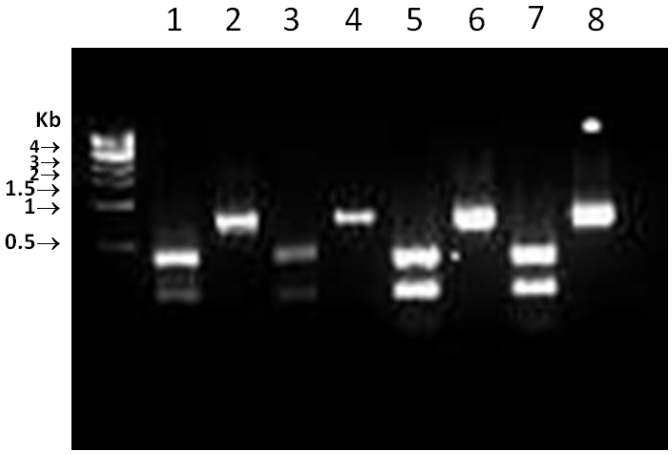

Fig.3. Ethidium bromide stained (2%) agarose gel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *AluI*. First comb lanes 2,4,6 & 8 are digested camel scolices and lanes1,3,5 &7 are undigested camel scolices. Second comb lanes 2,4,6,8&10 are digested sheep scolices and lanes 1,3,5,7 &9 are undigested sheep scolices.

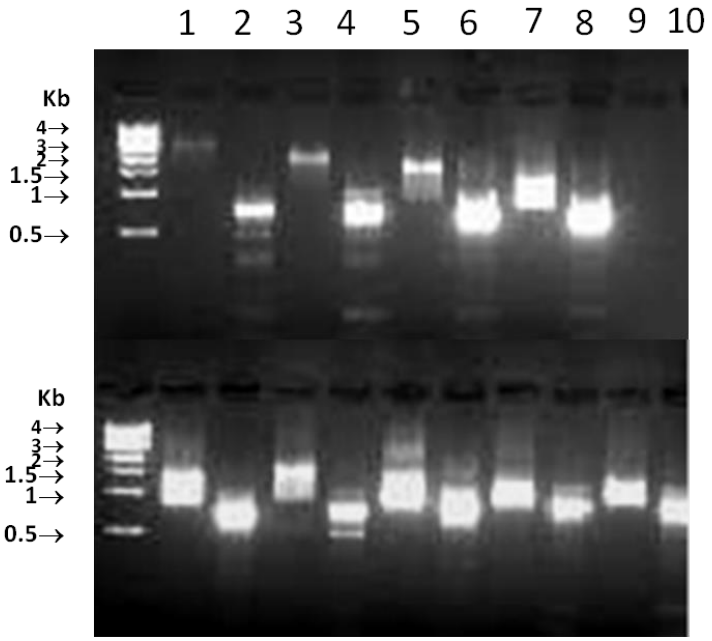

Fig.4. Ethidium bromide stained (2%) agarose gel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *AluI*. Lanes 2,4 & 6 are digested goat scolices and lanes1,3,5, &7 are undigested goat scolices.

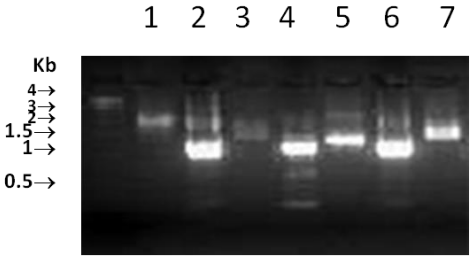

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Fig.5. Ethidium bromide stained (2%) agarose gel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *MspI*. First comb lanes 2,4,6 & 8 are undigested camel scolices and lanes1,3,5 &7 are digested camel scolices. Second comb lanes 2,4,6,8&10 are undigested sheep scolices and lanes1,3,5,7 &9 are digested sheep scolices.

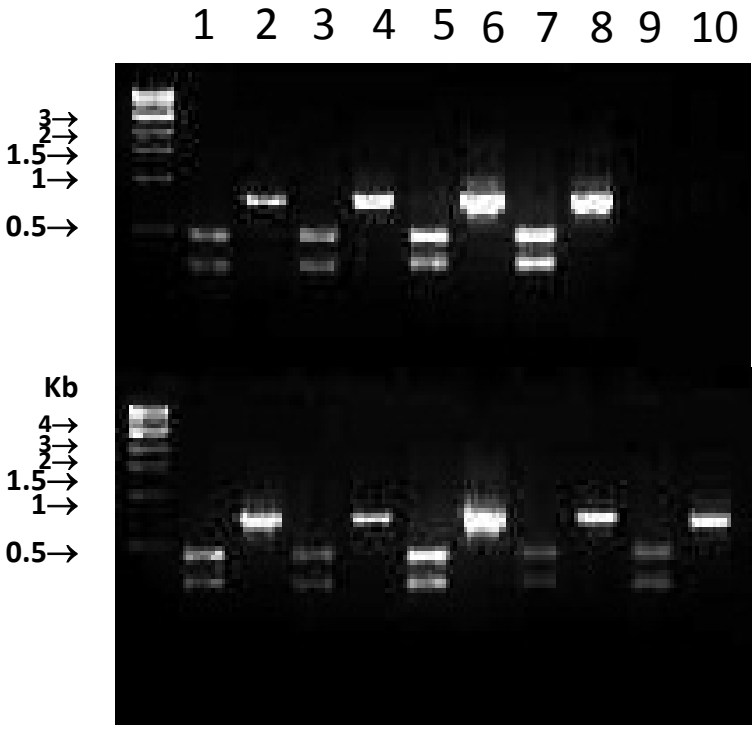

Fig.6. Ethidium bromide stained (2%) agarose gel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *MspI*. Lanes 2,4,6 &8 are undigested goat scolices and lanes1,3,5 &7, are digested goat scolices.

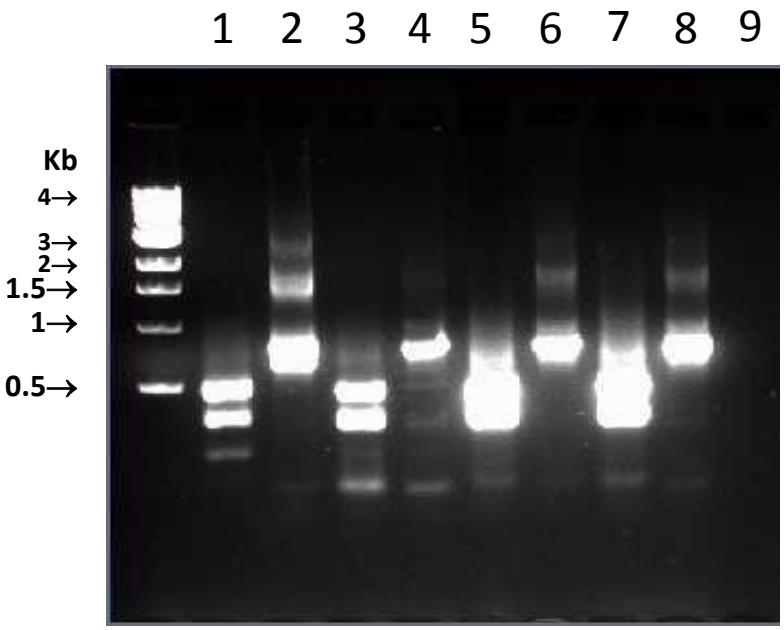

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Fig.7. Ethidium bromide stained (2%) agarosegel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *RsaI*. First comb lanes 2,4,6&8 are undigested camel scolices and lanes 1,3,5 &7 are digested camel scolices. Second comb lanes 2,4,6,8&10 are undigested sheep scolices and lanes 1,3,5,7 &9 are digested sheep scolices.

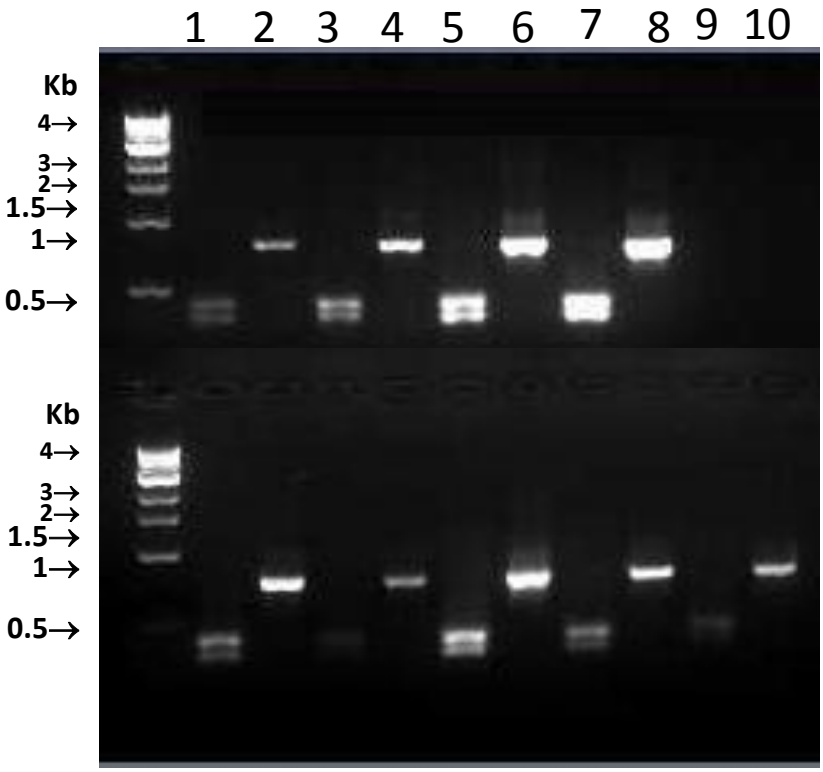

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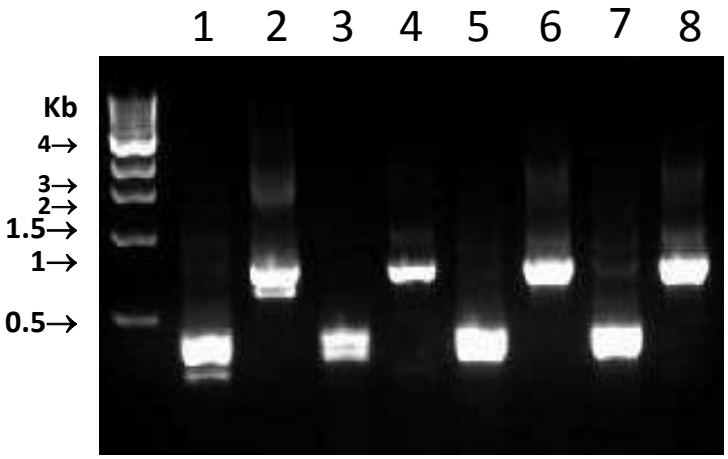
Fig.8. Ethidium bromide stained (2%) agarose gel showing amplicons of internal transcribed spacer gene 1 (ITS1) of *Echinococcus granulosus* after digestion with *RsaI*. Lanes 2,4,6 &8 are undigested goat scolices and lanes 1,3,5 &7 are digested goat scolices.



DISCUSSION

In Saudi Arabia, the camel, sheep and goat considered an important livestock which play a role in the income and the main source of milk and meat. Saudi camel, sheep and goat isolate of *E. granulosus* has been characterized earlier.

It has been reported that there are differences in some studied genes among several species, one of them the genotype variation in *E. granulosus* (Shahnazi et al., 2011). There are studies reported the existence of different strains from different animals and others gave identical patterns which may influence on a lot of factors which control the life cycle patterns, host specificity, development rate, antigenicity, transmission dynamics, sensitivity to chemotherapeutic agents and pathology of such organism. Since several years extensive literature on the application of molecular biological methods has been published in order to discriminate *Echinococcus* strains/species (McManus, 2006; Craig et al., 2007; Utuk et al., 2008).

The present work is considered the first study about the RAPD analysis amplification profiles obtained from twenty primers to compare the genotype of DNA isolates from *E. granulosus* extracted from camel, sheep and goat in Saudi Arabia.

The RAPD-patterns of all camel, sheep, goat isolates were identical; the result may be affected by several factors like the quality of extracted DNA and the program plan of the PCR (Siles-Lucas et al., 1993; Rinder et al., 1997). For this reason the result of RAPD analysis should be confirmed by one or more other DNA techniques. So, PCR-RFLP analysis is used to give more information about identification and variability of *E. granulosus* (McManus, 2002). Our work showed that the result of RFLP patterns of all camel, sheep and goat isolates were also identical.

Several studies have been published about the molecular characterization of *E. granulosus* isolated from different places in the world; the majority of it used PCR-RFLP analysis, e.g. from Mexico (Maravilla et al., 2004; Villalobos et al., 2007), Sardinia (Varcasia et al., 2006), Iran (Harandi et al., 2002; Ahmadi and Dalimi, 2006; Shahnazi et al., 2011; Khademvatan et al., 2013), Turkey (Utuk et al., 2008), India (Bhattacharya et al., 2008), Tunisia (M'rad et al., 2005), Argentina (Rosenzvit et al., 1999), Australia (Gasser and Chilton, 1995) and Slovakia (Sna´bel et al., 2000). Our result agreed with Bowles and McManus (1993) who reported the existence of different strains of *E. granulosus* in sheep, camel, horse, cattle and pig. All "sheep strain" (G1) isolates examined gave identical RFLP patterns and disagreed with other studies which reported that there are a variation in the genotype in different isolates form different hosts. Sheep and human isolates belonged to same genotype while camel isolates belonged to a different genotype (Ahmadi and Dalimi, 2006). To the best of our knowledge, there is no reports on the strain characteristics of *E. granulosus* in Saudi Arabia have been published. In this study, the results of RAPD were further tested by *ITS1*-RFLP for the characterization of *E. granulosus* samples taken from cysts infesting camel, sheep and goat inhabiting Saudi Arabia. As such, no *ITS-1* variant could be detected by *Rsa1*, *HhaI*, *MspI*, *AluI*. The result is indicative of absence of *ITS-1* variant which could not be discriminated by using these four restriction enzymes.

The echinococcosis still remains a major public health problem in Saudi Arabia and the hygiene practices and stricter control of animal slaughter one of the most important factors which play a great role in this problem. The present study is the first comprehensive genotypic analysis of *E. granulosus* infecting camel, sheep and goat intermediate hosts by using RAPD and PCR-RFLP analysis in Saudi Arabia.

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REFERENCES

Ahmadi, N. (2005). Hydatidosis in camels (*Camelus dromedarius*) and their potential role in the epidemiology of *Echinococcus granulosus* in Iran. *J Helminthol.*, **79**: 119–125.

Ahmadi, N. & Dalimi, A. (2006). Characterization of *Echinococcus granulosus* isolates from human, sheep and camel in Iran. *Infect. Genet. Evol.*, **6**: 85–90.

Arbabi, M., Massoud, J., Dalimi-Asl ,A. & Sadjjadi, S. (1998). Prevalence of hydatidosis in laughtered animals in Hamedan. *Daneshvar Sci Res J Shahed University.*, **5**: 57–61.

Bhattacharya, D., Bera, A. K., Bera, B. C., Pan, D. & Das, S. K. (2008). Molecular appraisal of Indian animal isolates of *Echinococcus granulosus. Ind J Med Res.*, **127**: 383-387.

Bowles, J., Blair, D. & Mcmanus, D. P. (1992). Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochemi Parasitol.*, **54**: 165–173.

Bowles, J., Blair, D. & Mcmanus, D. P. (1994). Molecular genetic characterization of the cervid strain ('northern form') of *Echinococcus granulosus. Parasitol.*, **109**: 215–221.

Bowles, J. & Mcmanus, D. P. (1993). Rapid discrimination of E*chinococcus* species and strain using a PCR-RFLP method. *Mol. Biochem. Parasitol.*, **57**: 231–239.

Craig, P. S., Mcmanus, D. P., Lightowlers, M. W., Chabalgoity, J. A., Garcia, H. H., Gavidia, C.M., Gilman, R.H., Gonzalez, A.E., Lorca, M., Naquira, C., Nieto, A. & Schantz, P.M. (2007). Prevention and control of cystic echinococcosis. *Lancet Infect. Dis.*, **7**: 85–394.

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

Gasser, R. B. & Chilton, N. B. (1995). Characterisation of taeniid cestode species by PCR RFLP of ITS2 ribosomal DNA. *Acta Trop.*, **59**: 31–40.

Harandi, M. F., Hobbs, R. P., Adams, P. J., Mobedi, I., Morgan-Ryan, U. M. & Thompson, R. C .A. (2002). Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran. *Parasitol.*, **125**: 367–373.

Khademvatan, S., Yousefi, E., Rafiei, A., Rahdar, M., & Saki, J. (2013). Molecular characterization of livestock and human isolates of Echinococcus granulosus from south-west Iran. *J Helminthol* ., 87:240-244.

Lavikainen, A., Lehtinen, M. J., Meri, T., Hirvela-Koski, V. & Meri, S. (2003). Molecular genetic characterization of the Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus. Parasitol.*, **127**: 207–215.

Maravilla, P., Thompson, R. C. A., Palacios-Ruiz, J. A., Estcourt, A., Ramirez-Solis, E., Mondragon-De-La-Pe˜Na, C., Moreno-Moller, M., Cardenas-Mejia, A., Mata- Miranda, P., Aguirre-Alcantara, M.T., Bonilla-Rodriguez, C. & Flisser, A. (2004). *Echinococcus granulosus* cattle strain identification in an autochthonous case of cystic echinococcosis in central Mexico. *Acta Trop.*, **92**: 231–236.

McManus, D. P. (2002). The molecular epidemiology of *Echinococcus granulosus* and cystic hydatid disease. *Trans Roy Soc Med Hyg.*, **96**: 151–157.

McManus, D. P. (2006). Molecular characterization of taeniid cestodes. *Parasitol. Int.*, **55**: 31–37.

M'rad, S., Filisetti, D., Oudni, M., Mekki, M., Belguith, M., Nouri, A., Sayadi, T., Lahmar, S., Candolfi, E., Azaiez, R., Mezhoud, H. & Babba, H. (2005). Molecular evidence of ovine (G1) and camel (G6) strains of *Echinococcus granulosus* in Tunisia and putative role of cattle in human contamination. *Vet. Parasitol.*, **129**: 267–272

Nei, M. & Li, W. H. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA.*, **76**: 5269-5273.

Rinder, H., Rausch, R. L., Takahashi, K., Koop, H., Thomschke, A. & Loscher, T. (1997). Limited range of genetic variation in *Echinococcus multilocularis. J Parasitol.*, **83**: 1045-1050.

Rokni, M. (2009) Echinococcosis/hydatidosis in Iran. *Iran J Parasitol.*, **4**:1–16.

Rosenzvit, M. C., Zhang, L. H., Kamenetzky, L., Canova, S. G., Guarnera, E. A. An D Mcmanus, D.P. (1999 Genetic variation and epidemiology of *Echinococcus granulosus* in Argentina. *Parasitol.*, **118**: 523–530

Saarma, U., Jogisalu, I., Moks, E., Varcasia, A., Lavikainen, A. Oksanen, A., Simsek, S., Andresiuk, V., Denegri, G., Gonzalez, L. M., Ferrer, E., Garate, T., Rinaldi, L. & Marvilla, P. (2009) A novel phylogeny for the genus *Echinococcus*, based on nuclear data, challenges relationships based on mitochondrial evidence. *Parasitol.*,**136** : 317–328.

Sambrook, J. & Russel, D.W. (2001). *Molecular cloning: A laboratory manual* Cold Spring Harbor Laboratory , New York. Pp. 6.28-6.30.

Scott, J .C., Stefaniak, J., Pawlowski, Z. S. & Mcmanus, D. P. (1997). Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus. Parasitol.*, **114**: 37–43

Shahnazia, M., Hejazib, H., Salehic, M. & Andalibd, A. R. (2011). Molecular characterization of human and animal *Echinococcus granulosus* isolates in Isfahan. *Iran Acta Trop.*, **117**: 47–50.

Sharbatkhori, M., Mirhendi, H., Fasihi Harandi, M., Rezaeian, M., Mohebali, M. Eshraghian, M.R., Rahimi, H. & Kia, E.B. (2010). *Echinococcus granulosus* genotypes in livestock of Iran indicating high frequency of G1 genotype in camels. *Exp Parasitol.*,**124**: 373–379.

Shareif , M. (2000). The prevalence of hydatid cyst infection in slaughtered animals. *J Shahid Sadoughi University of Med Sci and Health Services.*, **4**: 80–84.

Siles-Lucas, M., Cuesta-Bandera, C. & Cuesta-Bernito, M. (1993). Random amplified polymorphic DNA technique for speciation in studies of *Echinococcus granulosus. Parasitol Res.*, **79**: 343-345.

Sna'bel, V., D'amelio, S., Mathiopoulos, K., Turcekova´, L. & Ubinsky´, P. (2000). Molecular evidence for the presence of a G7 genotype of *Echinococcus granulosus* in Slovakia. *J. Helminthol.*, **74** : 177–181.

Thompson, R. C. A. (2008). The taxonomy, phylogeny and transmission of *Echinococcus. Exp Parasitol.*, **119**: 439–446.

Thompson, R. C., Lymbery, A. J. &And Constantine, C. C. (1995). Variation in *Echinococcus* towards a taxonomic revision of the genus. *Adv Parasitol.*, **35**: 145–176.

Utuka, A. E., Simsekb, S., Koroglub, E. & Mcmanusc, D. P. (2008). Molecular genetic characterization of different isolates of *Echinococcus granulosus* in east and southeast regions of Turkey. *Acta Trop.*, **107**: 192–194.

Varcasia, A., Canu, S., Lightowlers, M. W., Scala, A. & Garippa, G. (2006). Molecular characterization of *Echinococcus granulosus* strains in Sardinia. *Parasitol. Res.*, **98**: 273–277.

Villalobos, N., Gonzalez, L. M., Morales, J., De Aluja, A .S., Jimenez, M. I. Blanco, M.A., Harrison, L.J.S., Parkhouse, R.M.E. & Garate, T. (2007). Molecular identification of *Echinococcus granulosus* genotypes (G1 and G7) isolated from pigs in Mexico. *Vet. Parasitol.*, **147**: 185–189.

WHO/OIE Manual, (2002). Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern.