African Journal of Biotechnology Vol. 10(29), pp. 5563-5570, 22 June, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.919
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Integration of random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) and DNA sequencing in search for strain-specific pharmacological targets in *Echinococcus granulosus*

Kennedy Mwambete[1] and Hildebert B. Maurice[2]*

[1]Department of Pharmaceutical Microbiology, School of Pharmacy, Muhimbili University of Health and Allied, P.O. Box 65013, Dar es Salaam, Tanzania.
[2]School of Pharmaceutical Sciences, Saint John's University of Tanzania, Tanzania.

Accepted 18 March, 2011

Echinococcus granulosus **a parasite characterized with intra-species variability and genetic studies show existence of 10 genotypes (G1-G10). Host specificity and different susceptibility to intermediate hosts has also been demonstrated. Better understanding of this parasitosis can assist in designing appropriate control and preventive measures and its management based on strain-molecular peculiarity. Thus, the necessity for identification and characterization of all strains. A total of 96 hydatid cysts from which either protoscolces or germinal membranes were extracted followed by DNA extraction and then, amplification by random amplification of polymorphic DNA (RAPD). The RAPD products with distinctive bands were cloned in pGEMT-Easy vector and recombinant DNA subjected to sequencing. Twelve oligonucleotides primers were designed from recombinant DNA sequences and strains-specific PCR were conducted. The PRC products amplified by primers P1F2R2 (Ta=66°C, 35 cycles) and P1F1R1TX-PCRs (Ta = 66°C, 35 cycles) showed specie-specificity. Analysis of the DNA sequences showed homologies to some important molecules like laminin-binding protein and *g*lutathione transferase. Notwithstanding that the present study indicates partial success on attaining distinctive strain-specific DNA sequences, the resultant polymerase chain reaction (PCR) products were not strain-discriminatory. It is speculated that incorporation of more restriction endonucleases and well-adjusted reaction conditions strain-distinctive PCR-restriction fragment lenght polymorphism (RFLP) can be designed.**

Key words: *Echinococcus granulosus*, strain-specificity, random amplification of polymorphic DNA-polymerase chain reaction RAPD-PCR, DNA sequencing.

INTRODUCTION

Hydatidosis or cystic echinococcosis is a parasite zoonotic infection caused by cestodes of the genus *Echinococcus*. The genus *Echinococcus* contains four recognized species: *Echinococcus granulosus*, *Echinococcus multilocularis, Echinococcus vogel* and *Echinococcus oligarthrus*. These are readily distinguishable at both adult and larval stages by standard taxonomic criteria including host preferences, epidemiological, biological development and morphological characteristics (Dubinský et al., 1998; Karimi and Dianatpour, 2008). Several strains of *E. granulosus* have been proposed based upon those criteria (Siles-Lucas et al., 1996; M'radi et al., 2010). Human parasitism is mainly due to cystic form of *E. granulosus,* which also affects angulated domestic animals such as cattle, sheep,

*Corresponding author. E-mail: hildebert1@yahoo.com.

Abbreviations: RAPD, Random amplified polymorphic DNA; **RFLP,** restriction length polymorphism; **PCR,** polymerase chain reaction; **CO1,** cytochrome c oxidase subunit 1; **ND1,** nicotinamide adenine dinucleotide dehydrogenase 1; **DHs,** definite hosts; **His,** intermediate hosts; **GM,** germinal membrane; **NAP,** nucleotide-amino acid alignment program; **LAP,** local alignment program; **GSH,** glutathione; PPs, Peyer's patches; HCs, sensory hair cells; **EMBL,** European Molecular Biology Laboratory.

goats and horses.

The parasite's life cycle involves two hosts including definitive hosts (dogs or other carnivores) and intermediate hosts (the angulated domestic animals, human inclusive). In definitive hosts (DHs), damage caused by the adult worm is limited to anchorage site in the intestine of the DH, which causes necrosis to cells adhering to the parasite's suckers or rupture of Lieberkhun crypts, releasing host cells in the crypt. Massive infestations can provoke excessive production of mucus, but generally, does not tend to produce damage or important cellular reactions against the parasite (Siles-Lucas et al., 1996). In intermediate hosts (IHs), the parasite in form of metacestode (hydatid cyst-HC) is characterized by its typical three membranous layers namely, adventitious, laminar and germinal membranes. From the germinal membrane, brood capsules develop, each containing one or several invaginated heads (protoscoleces-PPs) that can develop into the adult tapeworm upon ingestion by the DH (Macpherson, 1983).

In order to kill the living germinal membrane (GM) and its contents-protoescoleces within the HC, the anthelminthic drugs must penetrate all the three barrier-layers, hence, comes the variability in efficacy of chemotherapeutic agents (Taylor et al., 1989; Teggi et al., 1993; Hildreth and Granholm, 2003). Thus, there is a pressing need of designing an appropriate and effective therapy. One of the several possible approaches is by conducting thorough genetic and molecular identification and characterization of strains, which in turn may lead to better understanding of targeted therapy (Duncan, 1997; Debouck and Goodfellow, 1999).

Moreover, *E. granulosus* is a parasite that shows a great intra-species variability all over the world. Genetic studies on *E. granulosus* have been carried out in several countries, demonstrating the existence of various strains categorized in 10 genotypes (G1-G10): Firstly, that which affects sheep (G1), cows (G2) and humans (G1); the second occurs in sheep, goat and human (G1), horses (G4), camel (G6), pig (G7), sheep Tasmania (G2) and cattle (G5); as well as buffalo (G3) and cervid (G8)-(González et al., 2002; Mwambete et al., 2004; M'radi et al., 2010). Genotypes G9 and G10 are still under study (Lavikainen et al., 2003; Busi et al., 2007; Vural et al., 2008). Host specificity and different susceptibility with regard to IHs has been demonstrated both in *in vivo* and *in vitro* studies Ponce-Gordo, 1995. Consequently, better knowledge on the disease will not only improve human health by designing appro-priate measures for its management based on involved strains, but also design chemotherapeutic agents based on differential molecular peculiarity with lesser side effects. Thus, there is a necessity to identify and charac-terize of all strains of this parasite.

Various techniques and approaches have been employed in studying this variability, naming: Random amplified polymorphic DNA (RAPD), restriction length and PCR-RFLP (González et al., 2002; Karimi and Dianatpour, 2008). Recently, sequencing of the parasite genomic DNA has been adopted (Mwambete et al., 2006. The sequencing of the mitochondrial cyto-chrome c oxidase subunit 1 (CO1) and NADH dehydro-genase 1 (ND1) genes have been extensively used as bench marks for molecular genetic characterization of parasites using these two primers (González et al., 2002; Mwambete et al., 2004).

MATERIALS AND METHODS

Biological parasite materials

Hydatid cysts were obtained from abattoirs and hospitals in the Autonomic Community of Castilla-Leone (Spain) derived from sheep, goats, horses, pigs and humans. Parasite materials employed comprise of Peyer's patches (PPs) or GMs obtained from the sensory hair cells (HCs) proceeded with aspiration of the fluids to reduce the cystic pressure. HCs need to be aseptically conserved and transported in appropriate containers to avoid contaminations. The obtained materials were stored at 4°C if were to be used within short time or at -20°C till further use.

Extraction and amplification of DNA

Phenol-chloroform-isoamyl standard procedures were used for the extraction of the genomic DNA followed by concentration and spectrophotometric quantification at 260 nm (25 Heath, 1997). RAPD reactions were conducted in 25 to 30 µl volumes containing 50 mM MgCl2, 25 mM dNTPs, 1% 20x Taq DNA polymerase buffer, 25 to 30 ng genomic DNA and 5 units of Taq DNA polymerase (Epicentre-Technologies, Spain). The following four primers consisting of 10 nucleotides each, were used at final concentration of 0.2 µM: 5;-GTT TCG CTC C-3' (OPB1), 5'-CTG CTG GGA C-3' (OPB10), 5'-CAG CAC CGC A-3' (OPZ3), 5'-CAA AGG GCG G-3' (OPAD1), and 5'-GGG AAT TCC C-3' (OPF6). The mixture was overlaid with 30 µl mineral oil (Sigma) prior to subjecting to thermocycler (GTC-2 Precision-Scientific, USA).

Conditions of reaction for RAPD were adjusted to: Initial denaturization at 94°C for 6 min, followed by 44 cycles at 94°C, 1 min at 36°C, 2 min at 72°C and final extension of 6 min at 72°C. The amplification products were separated in high temperature gelification agarose gels (2%) and visualized by ethidium bromide staining under UV illumination. These products may be directly subjected to sequencing of a distinctive fragment and/or to enzymatic digestion by restriction length enzymes/endonucleases.

Cloning of RAPD products

Strain-discriminating RAPD amplification products (Figure 1) from which 1 ul aliquot was drawn were subjected to cloning process using cloning vectors like pGEMT-Easy and thus, obtaining recombinants plasmids, which were selected accordingly (Promega, 2009). Recombinants plasmids were also processed by electrophoresis or /and RFLP in order to select DNA fragments of different sizes, though this does not guarantee sequences' compositions distinction. A plasmid that produced a single band or at least two brighter and wider band was considered to be the ideal insert/recombinant DNA. This process also served as means of quantifying the recombinants DNA prior to sequencing of the same (Duarte and Izquierdo, 1992; Health, 1997).

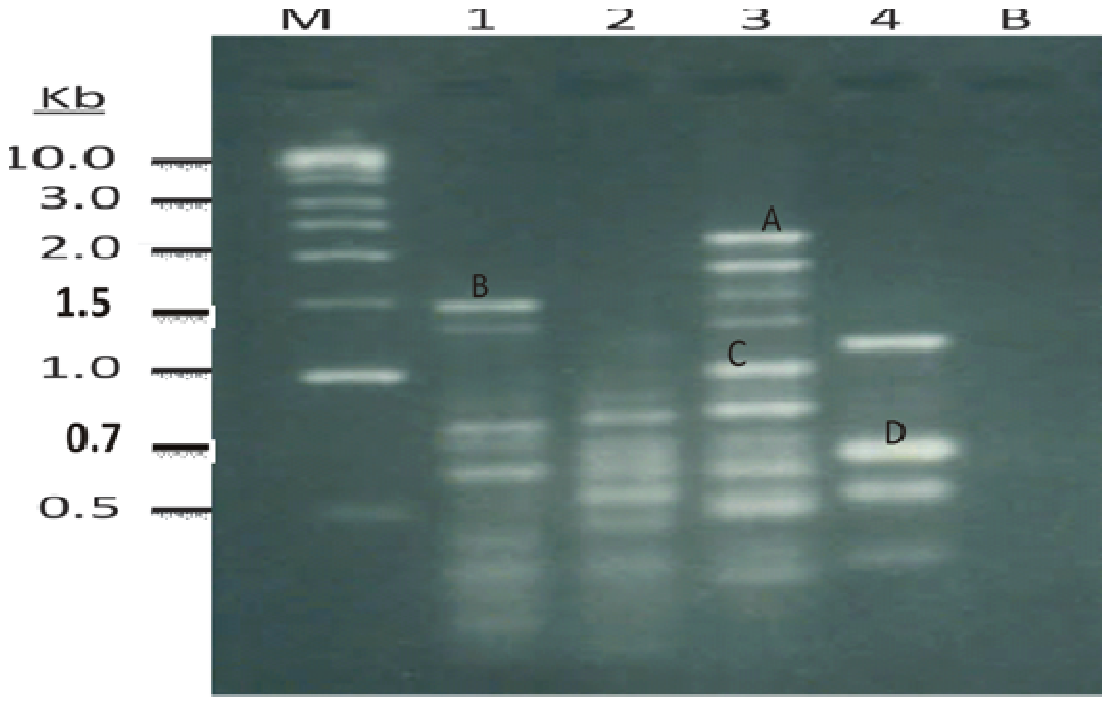

Figure 1. RAPD product with distinctive band patterns amplified by OPB1 primer. M, Molecular marker; 1, horse; 2, pig; 3, human, 4, sheep isolates and B-blank.

DNA sequencing and design of olygonucleotide primers

The parasite DNA inserts from recombinant plasmids were sequenced by fluorescence-based labelling sequencier ABI–PRISM-377 (Perkin–Elmer, Germany) which proceeded with comparison of DNA sequences using European Molecular Biology Laboratory (EMBL) and GenBank databases with other finder partners like the genetic computer software (Devereux et al., 1984, Rice et al., 2000). DNASTAR (England) was largely employed for analysis of DNA sequences by the primer select lasergene program and design of primers. Several factors were taken into consideration prior to selecting a given DNA sequence/fragment as a target for primers designing like positioning of the primer within the target template, melting temperature (Tm) and length of the fragment (mers) and most importantly score of the candidate primers. Primers that started with G and/or C or at least the second base were G or C; they were selected because of their stability and thus reproducibility of their PCR products. However, the G + C content ranging from 50 to 55% and Tm > 45°C produced better results than those with lower Tm.

Conditions of reactions for distinctive RAPD-PCR protocols

The H41F1R1-PCR (Ta = 60 to 66°C, 35 cycles) only amplified DNA from parasites of the genus *Echninococcus* without amplifying even the closely related genius of *Taenia* or other parasites of the family *Taenidae* to which *E. granulosus* belongs. Similarly, S6F1R1-PCR (Ta = 66°C, 35 cycles) amplified both isolates of *E. granulosus* and *E. multilocularis*.

Screening for proteins of clinical interests

Prior to nucleotide-proteins alignment, all DNA recombinants were checked for vector contaminationusing Basic Local Alignment Sequences Tool (BLAST) 2EVEC (Gish and States, 1993). Then, all sequenced DNA fragments were compared with already available EMBL and GenBank. DNA databases using various online software like advanced BLAST-(Gish and States, 1993), Washington University BLAST (WU-BLAST), nucleotide-amino acid alignment program (NAP) and local alignment program (LAP). The latter two programs served for ruling in or out presence of introns and frame shifts (Huang and Zhang, 1996; Huang et al., 1997).

RESULTS AND DISCUSSION

A total of 96 hydatid cysts from which either PPs or GMs were collected and subsequently employed for DNA extraction were gotten. Figure 2 shows the OPB1 primer RAPD amplified DNA (derived from horse, pig, sheep, goat and human) with clear distinctive band patterns among them at A(2.0 kb), B (1.5 kb), C(1.0 kb) and D(0.7 kb), which were later cloned in the pGEMT-Easy vector. The cloned RADP products resulted into 23 recombinant DNA/inserts that were in turn, primed by DNAStar (PrimerSelect) software, 12 potential pairs of olygo-nucleotide primers were identified by using SeqMan program (DNAStar, UK) as depicted in Table 1. However, only 4 pairs of primers (H41F1R1, S6F1R1, P1F2R2 and P1F2R1TX) derived from 4 DNA sequenced inserts (H41, S6, P1a and P1b) were designed (Figure 2).

Specie-specificity was demonstrated by the primers P1F2R2 (Ta=66°C, 35 cycles) and P1F1R1TX-PCRs (Ta = 66°C, 35 cycles), which actually were specific to the

A

GTTTCGCTCCCAGTTGATGTGGGTTAAAACAGATTGTTTTATCATCCCTCAAATTAAACA 60
CAGGTAAAATTTGCTCTAGGCAAACTAATCGACAATAACTTTTTCTCATGATGACGTCAT 120
TCTTCATCCACGTTAAAACTAAATGGTTCTCTCTTCTTAGCTTGCTCCATCTACCTTTGA 180
GTGGTTCATCACCCGAGTCAAAATACTTATTAAAACATTTGATTTTTGAGCAGCACGTTG 240
TTCCACTACCGCATACCGCGCCTTCTTTAAAGGTTTGGCAACAAGTTGTAGGAGTAACTA 300
GCTCCTACGGCTTTGTAAGCCAATGAAACTCGGTACGTAGAGCAGCAGACAAGTTCAAAT 360
CACATCCACCAGGTTCAACTGACATTTTATTATAAAAGTTGAAAAATAACAGAGCACAAT 420
AACGAGTACCATACCGAGAGACTGACGGATAACTCCACCTTATAATGCATCCATACAAGC 480
AAAaCGCACGAAACTTTCTGGTCACGTGGTAATAAGAAGTAATTTTTTAAAACAGTCTCA 540
CATTAAATGAATTTTAGAAGTGCCTATAAGTTACAACGAGTCATTGTATCGAGGTGTCTA 600
AGCCCTTCCCTACACACCGACAGTCAAGCTGGATTCAGTGTGAGGGACTCTTTTgCAGTC 660
ACAAAAGTCATTCAAGCGTTGGC 683

B

TGTTTCGCTCCGTAGTAGCTTATCGGTTTAACAGGAGTACAAAGCATTTCTTCTCACCCC 60
AGAACTGTGTAGACATAGAAATGCCCCAAGGCCCAACCAGATACCTCTGAACCCGTAGTT 120
TTTCTGCCTCCCCAAGTTATTTGCTAGACCTTTGAgTCGAATGGTGACATGAACATTAAG 180
TTCGAGCTCCGGAAAATTTAAAACGAATGAATACCATGCACAATCAGAAGATGAgCAAAG 240
TTTGCAACGCAATTTTGCAATTTGGAAAGAAACACAATGAAACTCCGATGACTAAACATG 300
CATCGCACCCACCGTCTCTGAAATATTCTATTGCTCATAGCTGATAAATTCACAACGCCG 360
TTACTGATGCGTGGGAAAGTATCATTAAGGCGTGACTGACTTTCCCAAATGGATAGGCAA 420
CAACCTGATGGCGGTGGGAAAGTATCATTAAGGCGTGACTGACTTTCCCAAATGGATAGG 480
CAACAACCTGTGCTAACTTTGGAAAAGGTGAGTTTTGTTATTTTGAATCCCAAAATCCCA 540
ATGTACAAAGTGTTTGACAGATATTAAAAAGTCGCATCGAATACAGCCCTTGTAATCCGC 600
TCTGAACGGTAGTACCGGCGGCTACTCACGTGACCTGTTCAAGTACAGCCTGATGCAGGC 660
TGAGTTCTCGTCCTATCGCATTGACTGTCACAATACATCCTTCACACGCACACACCGGGG 720
TATGGGTGTTGGACCGATGAACAGAAGGGTGGACATGGCAGGCGCTCACAGTGTCAGGCC 780
ATATTCTGAGAAAGACCTGAAAATTCCGAGGAAAGACCGGACACGGTCACTCGCCTCATT 800
CAATAACTGAGAAGAATAATCTTTGCAGGAGCGAAAC 877

C

GGGTTAAGCGGCCCTCGTACGCTGCAGCCCGGCCGCCATGGCCGCGGGATGTTTCGCTCC 60
TGTTTTAGTGTCGTTGCCTTTCCTTTTAAATGTTTTTTTTTTTCAGTACTTTTCTCATGC 120
TGTTGCTGCTGTCATGTGACACGTGATTGTGTAGACAATTGACAATTGCTTTGTAGCTCG 180
TCAAATGTCCAAACATAAGAGGATAGTTAGTTTTAATCAACCTTCTGATACTGATTTAAA 240
ATTGACAGGATCTTCATAATTGTTGCTGCCAGTTCCGATTAACTCCTTTAACAGCGTAGT 300
GAAACTGTTATGCTCTGTTGTAGGAGCAATAGTTTGGCATCGAAATTCGGATTGGGAATG 360
AGAAATTTGAATCCGATTAAAGTTGGGGCTCGAGTATTGCCACAACCGAGTGCATGCTTT 420
GGCGGTTGGTCGATGAAACTTGATAGGGGGAGCTGGTACCAGGGGCCATTCACAAACCCG 480
R1TX R2
TACCTTTTTTGAGATGCGCAGTCATCGTCATGGTACCGGTGATGATGTAAGACTTTAAGG 540
TGCTCGCTTATTGCAGTAgGTCTGAGTGCTTGAGGTCATCTCTGAAAGTTAGTATTTGGT 600
TTGGTTGGATATTCCGCTCGAGAGATTGCAGTTTTAATTGGACAATTGTGATTTTAATAA 660
ACCACAAATGCTAAAAATTAT 681

Figure 2. Olygonucleotide primers' sequences for RAPD-PCR (Panels A TO C): Panel A, H41 plasmid (primers: F1 and R1); panel B, S6 plasmid (primers: F1 and R1) S6F1R1; panel C: P1a andP1b plasmids (primers F2 + R2, and F2 + R1TX, respectively) P1F2R2/P1F2R1TX.

Table 1. Some chemical and physical properties of the experimental soils before sowing.

Soil property		Soil depth	
		0 to 30 cm	30 to 60 cm
pH (1:2 soil: water)		7.25	10.45
Organic matter g kg^{-1}		18	6.65
$CaCO_3$ g kg–1		150	137.28
Total N g kg^{-1}		0.78	0.28
Available P mg kg^{-1}		9.3	3.28
Cation exchangeable capacity cmolc kg^{-1}		32.0	27.5
Exchangeable cations, cmolc kg^{-1}	Ca^{+2}	19.8	20.09
	Mg^{+2}	4.5	3.04
	K^{+1}	2.7	1.33
	Na^{+1}	0.15	0.10
Microelements, mg kg^{-1}	Fe^{+2}	3.36	1.32
	Mn^{+2}	1.74	1.17
	Zn^{+2}	1.75	1.14
	Cu^{+2}	0.78	0.69
	B	0.16	0.08
Electric conductivity dS m^{-1}		1.44	1.05
Soil particle size distribution	Clay %	32.6	27.5
	Silt %	42.3	40.4
	Sand %	25.1	32.1
Soil textural class		CL	L

extent that they could not amplify even DNA derived from isolates of the same genus (*E. multilocularis*) nor equine isolates of *E. granulosus* that is phylogenetically considered as very distinct strains (Not shown). Nevertheless, these results could not distinguish strains of *E. granulosus* but identify strain-specific DNA fragments.

Results obtained from DNA sequences database showed homologies to some important proteins/ molecules: Laminin-binding protein (from clone S6), which belongs to the family of multifunctional egmo proteins in *E. granulosus* and glutathione (GSH) transferase was transcribed from clone S3 (sheep origin) as shown in Table 2.

The H41 recombinant DNA demonstrated 58% homology to sequence LV0233066 *Taenia solium* UNAM-cd2_larva cDNA, mRNA sequence a parasite in the same family. While P1a clone exhibits 62% similarity with *Caenor-habditis briggsae* contig cb25.fpc2888 from assembly cb25.agp8, accession number EMBL: CAAC-01000061.

In the present study, the PCR products amplified by the primers P1F2R2 (Ta=66°C, 35 cycles) and P1F1R1TX-PCRs (Ta = 66°C, 35 cycles) demonstrated to be specie-specific, thus, were unable to amplifyDNA derived from isolates of the same genus (*E. multilocularis*) nor equine isolates of *E. granulosus* that is phylogenetically considered as very distinct strains (Mwambete, 2002). Nevertheless, these results could not distinguish strains of *E. granulosus* but identify strain-specific DNA fragments that could be further explored and come up with molecules of clinical interests. Moreover, this could be employed as starting-point by other researchers who may utilize larger number of restriction enzymes and thus produce strain-specific DNA band profiles and thus, identifies the distinctive fragments thoroughly.

Proper diagnosis for hydatidosis and identification of each strain is of paramount importance, because of the differences in the parasite development, epidemiological characteristics and control measures (Schantz et al., 1995; Capuano et al., 2006). The main reason is the high manifested genetic variability of the parasite, which has its impact on chemotherapy as well (Hossein and Islami, 1998). Similarly, sometimes differential diagnosis is required to rule out other parasitic infections that resemble echninococcosis (George et al., 2004).

It is well known that reproducibility of the amplification

Table 2. Effect of boron applications on soil microbial population and CO_2-C production.

Soil	B	Microbial population									CO_2-C production		
Depth (cm)	Rate (kg da^{-1})	Bacteria			Fungi			Actinomycetes			CO_2-C		
		SP	FP	HP	SP	FP	HP	SP	FP	HP	SP	FP	HP
0-30	0	25.70 ^{b}	37.75 ^{b}	29.61 ^{ab}	36.16 ^{b}	57.03 ^{b}	46.77 ^{b}	16.99 ^{b}	22.25 ^{b}	16.65 ^{b}	6.48 ^{b}	9.94 ^{ab}	7.78 ^{b}
	1	31.42 ^{ab}	42.00 ^{ab}	30.36 ^{ab}	42.75 ^{ab}	67.30 ^{ab}	53.26 ^{ab}	17.33 ^{ab}	25.19 ^{ab}	19.85 ^{ab}	7.79 ^{ab}	11.52 ^{ab}	8.40 ^{ab}
	3	34.67 ^{a}	47.50 ^{a}	37.60 ^{a}	46.91 ^{a}	72.64 ^{a}	61.25 ^{a}	21.00 ^{a}	31.03 ^{a}	24.19 ^{a}	8.57 ^{a}	12.67 ^{a}	10.20 ^{a}
	6	25.37 ^{ab}	47.00 ^{a}	24.80 ^{b}	40.48 ^{ab}	72.00 ^{a}	59.12 ^{a}	17.66 ^{ab}	26.95 ^{ab}	20.46 ^{ab}	7.33 ^{ab}	12.55 ^{a}	8.26 ^{ab}
	9	13.34 ^{b}	30.40 ^{c}	16.90 ^{c}	25.44 ^{c}	43.63 ^{c}	35.68 ^{c}	11.66 ^{c}	14.71 ^{c}	11.43 ^{c}	4.96 ^{c}	7.70 ^{b}	4.94 ^{c}
Ave.		26.10 ^{b}	40.93 ^{a}	27.85 ^{b}	38.35 ^{c}	62.52 ^{a}	51.22 ^{b}	16.93 ^{b}	24.03 ^{a}	18.52 ^{b}	7.03 ^{b}	10.88 ^{a}	7.92 ^{b}
30-60	0	13.20 ^{b}	19.27 ^{b}	15.03 ^{ab}	52.10 ^{b}	76.29 ^{b}	59.67 ^{b}	25.93 ^{b}	42.39 ^{b}	33.19 ^{b}	2.71 ^{b}	4.15 ^{b}	3.42 ^{b}
	1	14.36 ^{a}	23.56 ^{a}	16.81 ^{ab}	60.14 ^{ab}	89.12 ^{ab}	63.98 ^{ab}	32.09 ^{a}	49.65 ^{ab}	36.81 ^{ab}	3.12 ^{a}	5.05 ^{a}	4.02 ^{ab}
	3	15.27 ^{a}	24.15 ^{a}	21.05 ^{a}	65.21 ^{a}	95.80 ^{a}	79.70 ^{a}	35.79 ^{a}	55.07 ^{a}	43.36 ^{a}	3.67 ^{a}	5.60 ^{a}	5.75 ^{a}
	6	14.00 ^{a}	24.00 ^{a}	19.79 ^{a}	57.37 ^{ab}	95.00 ^{a}	64.38 ^{ab}	29.93 ^{ab}	54.50 ^{a}	38.03 ^{ab}	3.23 ^{a}	5.21 ^{a}	3.92 ^{ab}
	9	8.34 ^{c}	14.57 ^{c}	11.23 ^{b}	39.02 ^{c}	59.54 ^{c}	39.36 ^{c}	18.89 ^{c}	32.02 ^{c}	21.29 ^{c}	2.45 ^{b}	3.29 ^{b}	2.74 ^{c}
Ave.		13.03 ^{c}	21.11 ^{a}	16.78 ^{b}	54.77 ^{c}	83.15 ^{a}	61.42 ^{b}	28.52 ^{c}	46.73 ^{a}	34.54 ^{b}	3.04 ^{c}	4.66 ^{a}	3.97 ^{b}

SP, Sowing period; FP, flowering period; HP, harvest period. Bacteria 10^{-6} CFU g^{-1} soil; Fungi 10^{-4} CFU g^{-1} soil; Actinomycetes 10^{-3} CFU g^{-1} soil, CO_2-C mg C m^{-2} h^{-1}.

reactions largely depend on specificity of primers and nature of nucleotides employed (Duarte and Izquierdo, 1992). Therefore, PCR and currently, DNA sequencing have taken the lead in this arena.

Likewise, DNA sequencing, preceded by RAPD-PCR has been utilized in screening for proteins of pharma-cological or/and diagnostic interests, using various online programs namely ExPASy-proteomics tool, which translate DNA sequences into proteins and functional websites for parasites genomes (Ivens et al., 2000). Thus, narrowing the screening for drug targets using genetic or molecular based approaches, now seem to be more appropriate than the traditional one which involve huge amounts of reagents/resources, time and person-nel. This proves to be useful, particularly *E. granulosus*, since its *in vitro* tests usually take long time, a minimum of 3 to 6 months, as result of slow *in vivo* and *in vitro* development of the parasite. In addition to that, the three protective hydatid cyst layers create another problem, as consequence of drug-permeability barrier and intrinsic drug solubility hindrance (Kammerer and Schantz, 1984; Hossein and Eslami, 1998).

Information obtained from the DNA databases regarding *E. granulosus* nucleotides homology to other organisms, may lead to discovery of common drug targets and thus, these other microorganisms might also be used as alternative experimental modules in searching for novel chemotherapeutic agents. Genetic variability of various organisms is a result of either acquired or intrinsic factors that are inevitable means for survival and adaptation to environmental changes and challenges, which unfortunately, occasionally become detrimental to human beings (Hartwell et al., 2000). Drugs resistance is one of these calamities that can be effectively monitored by molecular genetic surveillance, thus, coming up with both ideal diagnostic and chemotherapeutic measures. Understanding gene sequences is crucial step towards underlying the molecular mechanisms such as essential biological processes, transmission, pathogenesis and drug resistance as well as identification of new drug targets and vaccine design. Nevertheless, genomes may not easily translate into a comprehensive recognition of gene and their roles in parasites biology (Ferdig and Su, 2000; Zhao and Hamilton, 2007). In that instance, complementary methods might be required to define relationship between genetic variations that is, sequence

differences and their consequent functional effects.

Results obtained from DNA sequences database show homologies to some important proteins/molecules that can be further explored and come up with potential drug targets like laminin-binding protein (from clone S6), which belongs to a family of multifunctional egmo proteins in *E. granulosus*, is associated with cell division and growth (Zhang et al., 1997). Laminin-receptor seems to be associated with invasive capacity and pathogenesis in bacteria and fungi (Narasimhan et al., 1994). From clone H35, elongation factor -1 (EF-1) was also uncovered. The parasite shows a high conservation of amino acids at the conserved position for EF-1 hand calcium-binding sites (Rodrigues et al., 1997).

Glutathione (GSH) transferase was also transcribed from clone S3. Usually, the helminth GSH transferase is present as isoenzymes, although, does not elicit clear biochemical homology to any of the three mammalian GSH transferase families. The GSH have diverse functions including detoxification, binding a range of anthelminthics and lipid peroxidation (Brophy and Barrett, 1990). On the other hand, H41 recombinant DNA with 58% homology to sequence LV0233066 *T. solium* UNAM-cd2_larva cDNA, mRNA sequence, is a parasite in the same family that buttress the RAPD-PCR findings. P1a clone exhibits 62% similarity with *C. briggsae* contig cb25.fpc2888 from assembly cb25.agp8, accession number EMBL: CAAC01000061 (Gish and States, 1990).

It is clear that RAPD-PCR and DNA sequencing cannot be routinely performed, because of not being cost-effective; however, they may be used for monitoring drug efficacy and resistance. In the long-run, they are worth to be conducted. However, if they are ignored, it may lead to utilization of chemotherapeutic agents on already resistant organisms, thus, incurring unnecessary expenses. It is therefore suggested that, RAPD-PCR, DNA sequencing and other similar approaches need to be periodically employed for monitoring genetic-mole-cular variability, screening and designing novel molecules for targeted therapy on the altered/ mutated drug receptors.

Finally, better understanding of pathogens, pathogenesis and diagnosis may contribute to appropriate and timely therapy (Kiresi et al., 2003; Masroor et al., 2010). In order to attain this, integrated techniques and approaches in fighting this parasitosis are inevitable. Such strategy can certainly improve our health care systems and thus, save patients' lives and untimely deaths. In this case, not only human can benefit from the advance in science and technology by increased income and productivity. Consequently, effective exploration and validation of these targets may definitely lead to discovery of therapeutic targets against parasitic helminthic and microbial infections. Notwithstanding that the present study indicates partial success on attaining distinctive strain-specific DNA sequences, the resultants PCR products were not strain-discriminatory. It is speculated that incorporation of more restriction endonucleases and well-adjusted reaction conditions, a differential PCR-RFLP can be obtained. Similarly, an increase of HC sample size from distinct regions may lead to acquisition of diverse DNA molecules hence, exploring further on strains' genetic variability of the parasite.

REFERENCES

Brophy PM, Barrett J (1990). Glutathione transferase in helminths. Parasitol. 100: 345-349.

Busi M, Snábel V, Varcasia A (2007). Genetic variation within and between G1 and G3 genotypes of Echinococcus granulosus in Italy revealed by multilocus DNA sequencing. Vet. Parasitol. 150: 75-83.

Capuano F, Rinaldi L, Maurelli MP, Perugin AG, Veneziano V, Garippa G, Genchi C, Musella V, Cringoli G (2006). Cystic echinococcosis in water buffaloes: epidemiological survey and molecular evidence of ovine (G1) and buffalo (G3) strains. Vet. Parasitol.137 (3-4): 262-8.

Debouck C, Goodfellow N (1999). DNA microarrays in drug discovery and development. Nat. Genetics Suppl. 21: 48-50.

Devereux J, Haeberli P, Smithies O (1984). A comprehensive set of sequence analysis programs for the VAX. Wisconsin Genetics Computer Group software package, version 7.0. Nucleic Acids Res. 12: 387-395.

Duarte RG, Izquierdo M (1992). In: Ingeneria genetic, Ed. Pp. 73-98. Ediciones Piramides, Barcelona, Spain.

Dubinský P, Stefancikova A, Turčeková A, Macko JK, Soltys J (1998). Development and morphological variability of *Echinococcus granulosus*. Parasitol. Res. 84: 221-229.

Duncan R (1997). Drug Targeting: Where are we now and where are going? J. Drug Targeting, 5(1):1-4.

Ferdig MT, Su X-z (2000). Microsatelllite markers and genetic mapping in *Plasmodium falciparum*. Parasitol. Today, 16 (7): 307-312.

George S, Villard O, Filisetti D, Mathis A, Marcellin L, Hansmann Y, Candolfi E (2004). Usefulness of PCR Analysis for Diagnosis of Alveolar Echinococcosis with Unusual Localizations: Two Case Studies. J. Cl. Microbiol. 42(12): 5954-5956.

Gish W, States DJ (1993). Identification of protein coding regions by database similarity search. Nat. Genet. 3: 266-272.Available at: (http://blast.wustl.edu/blast).

González LM, Daniel Mwambete K, Montero E, Rosenzvit MC, Garate T. and Cuesta-Bandera C (2002). Further Molecular Discrimination of Spanish Strains of *E. granulosus*. Exp. Parasitol. 102: 46-56.

Hartwell LH, Hood L, Goldberg ML, Reynolds AE, Silver LM, Veres RC (2000). The genetic analysis of populations and how they evolve. In: Genetics-from genes to genomes. 23: 755-782. McGraw-Hill Higher Education, New York.

Heath S (1997). Molecular techniques in analytical parasitology. In: Rogan MT (Ed). Analytical Parasitol. (Springer Lab Manual), Springer-Verlag, New York, pp. 66-82.

Hildreth MB, Granholm NH (2003). Effect of mouse strain variations and cortisone treatment on the establishment and growth of primary Echinococcus multilocularis hydatid cysts. J. Parasitol. 89(3): 493-5.

Hossein SH, Eslami A (1998). Morphological and developmental characteristics of *Echinococcus granulosus* derived from sheep, cattle and camels in Iran. J. Helminthol. 72: 337-341.

Huang X, Zhang J (1996). Methods for comparing a DNA sequence with a protein sequence. Comput. Appl. Biol. Sci.12: 497-506.

Huang X, Adams MD, Zhou H. and Kerlavage AR (1997). A tool for analyzing and annotating genomic sequences. Genomics, 46: 37-45.

Ivens I, Aslett I, Wood I (2000). Functional Websites for Parasite Genome Projects. Parasitol. Today, 16: 93-94.

Kammerer WS, Schantz PM (1984). Long term follow up of human hydatid disease (*Echinococcus granulosus*) treated with a high-dose mebendazole regime. Ann. J. Trop. Med. Hyg. 33: 132-137.

Karimi A, Dianatpour R (2008). Genotypic and phynotypic of *Echninococcus granulosus* of Iran. Biotechnology, 7(4): 757-762.

Kiresi DA, Karabacakoğlu A, Odev K, Karaköse S (2003). Uncommon locations of hydatid cysts. Acta Radiol. 44: 622-636.

Lavikainen A, Lehtinen MJ, Meri T, Hirleva-Koski V, Meri S (2003).

Molecular genetic characyterization of the Fennascandian cervid strain, a new genotypic group (G10) of the *Echninococcus granulosus*. Parasitol. 127: 207-215.
Macpherson CNL (1983). An active intermediate host role for man in the life cycle of *Echinococcus granulosus* in Turkana, Kenya. Am. J. Trop. Med. Hyg. 32: 399-404.
Masroor I, Azeemuddin M, Khan S, Barakzai A (2010). Hydatid disease of the breast. Singapore Med. J. 51(4): 72-75.
M'rad S, Oudni-M'rad M, Filisetti D, Mekki M, Nouri A, Sayadi T, Candolfi E, Azaiez R, Mezhoud H, Babba H (2010). Molecular Identification of *Echinococcus granulosus* in Tunisia: First Record of the Buffalo Strain (G3) in Human and Bovine in the Country. Open Vet. Sci. J. 4: 27-30.
Mwambete DK, Alamo R, Cuesta-Bandera C, Ponce-Gordo P (2006). Molecular Survey of *Echinococcus granulosus* isolates from Castilla-Leon (Spain) from 1993-2002. Rev Iber. Parasitol. Extraordinary, (65): 3-4.
Mwambete DK, Ponce Gordo F, Cuesta Bandera C (2004).Genetic Identification and Host range of the Spanish strains of *Echinococcus granulosus*. Acta Trop. 91(2): 87-93.
Mwambete DK, Ponce-Gordo F. & Cuesta-Bandera C (2004). Genetic identification and host range of the Spanish strains of *Echinococcus granulosus*. Acta Trop. 91(2): 87-93.
Mwambete KD (2002). Cepas de *Echinococcus granulosus* en Castilla y León. Desarrollo de una PCR distintiva. Univeresidad Complutense de Madrid, PhD Thesis.
Narasimhan S, Armstrong MYK, Rhee K, Edman JC, Richards FF. and Spicer E (1994). Gene for an extracellular matrix receptor protein from *Pneumocyst carnii*. Proc. Natl. Acad. Sci. 91: 7440-7444.
Promega-(2009). pGEMT and pGEMT Easy Vector Systems. Technical manual of instructions. No. 042. USA.
Rice P, Longden I, Bleasby A (2000). EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet. 16: 276-77.
Rodrigues JJS, Ferreira HB, Farias SE and Zaha A (1997). A protein with a novel calcium-binding domain associated with calcareous corpuscles in *Echinococcus granulosus*. Biochem. Biophys. Res. Commun. 237: 451-456.
Schantz PM, Chai J, Craig PS, Eckert J, Jenkins DJ, Macpherson CNL, Thakur A(1995). Epidemiology and control of hydatid disease, in *Echinococcus* and Hydatid disease. Thompson RCA, Lymbery AJ (Ed.), CAB Intl. Wallingford pp. 233-350.
Siles-Lucas M, Benito M, Cuesta-Bandera C (1996). *Echinococcus granulosus*: genomic and isoenzymatic study of Spanish strains isolated from different intermediate hosts. Vet. Parasitol. 63: 273-282.
Taylor DH, Morris DL, Richards KS. (1989). *Echinococcus granulosus*: in vitro maintenance of whole cysts and the assessment of the effects of albendazole sulphoxide and praziquantel on the germinal layer. Trans. Royal Soc. Trop. Med. Hyg. 83: 535-538.
Teggi A, Lastilla MG, De Rosa F (1993).Therapy of human hydatid disease with mebendazole and albendazole. Antimicrob. Agents Chemother. 37(8): 1679-1684.
Vural G, Baca AU, Gauci CG, Bagci O, Gicik Y, Lightowlers MW (2008). Variability in the Echinococcus granulosus cytochrome C oxidase 1 mitochondrial gene sequence from livestock in Turkey and a re-appraisal of the G1-3 genotype cluster. Vet. Parasitol. 154(3-4): 347-50
Zhang L, Leggat GR, Kalima BH, Piva TJ, McManus DP (1997). Cloning and expression of c DNA encoding a nonintegrin mainin-binding protein from *E.granulosus* with localization of laminin-binding domain. Mol. Biochem. Parasitol. 87: 183-192.
Zhao C and Hamilton T (2007). Introns Regulate the Rate of Unstable mRNA Decay. J. Biol. Chem. 282: 20230-20237.