

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*

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*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 glutathione 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 length 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,

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**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-protoscoleces within the HC, the anthelmintic 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 appropriate 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 characterize 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 cytochrome c oxidase subunit 1 (CO1) and NADH dehydrogenase 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 Autonomous 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.

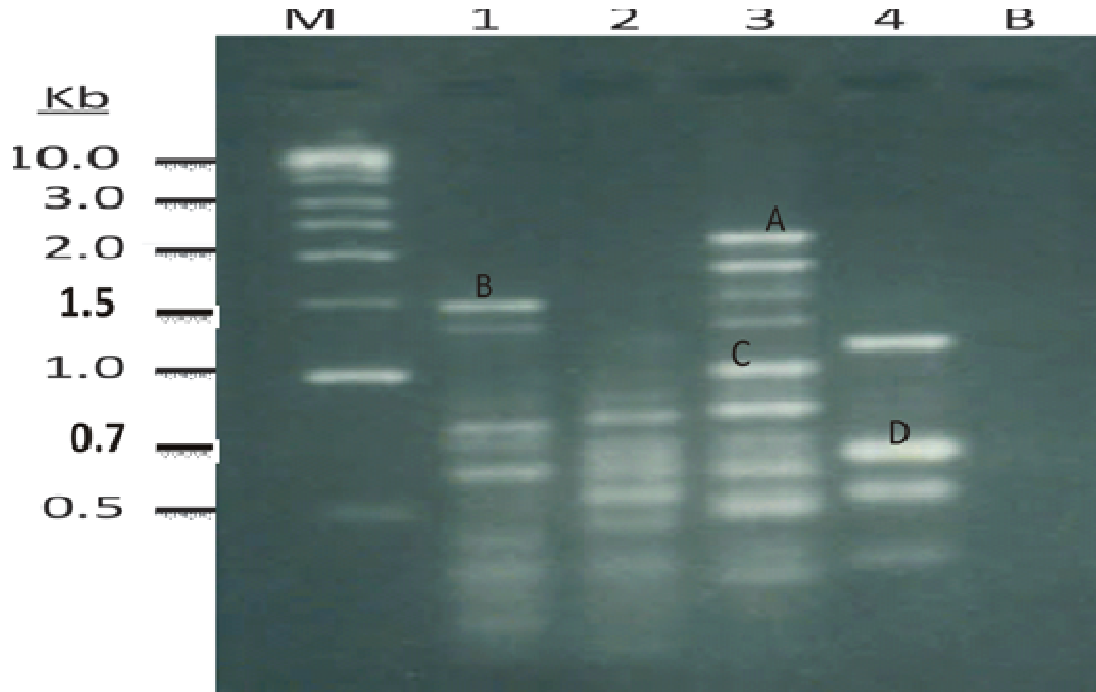
### Extraction and amplification of DNA

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 MgCl<sub>2</sub>, 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 denaturation 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 µl 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; Heath, 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 oligonucleotide primers

The parasite DNA inserts from recombinant plasmids were sequenced by fluorescence-based labelling sequencer 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 ( $T_m$ ) 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  $T_m > 45^\circ\text{C}$  produced better results than those with lower  $T_m$ .

#### Conditions of reactions for distinctive RAPD-PCR protocols

The H41F1R1-PCR ( $T_a = 60$  to  $66^\circ\text{C}$ , 35 cycles) only amplified DNA from parasites of the genus *Echinococcus* without amplifying even the closely related genus of *Taenia* or other parasites of the family *Taenidae* to which *E. granulosus* belongs. Similarly, S6F1R1-PCR ( $T_a = 66^\circ\text{C}$ , 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 contamination using 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 RAPD products resulted into 23 recombinant DNA/inserts that were in turn, primed by DNASTar (PrimerSelect) software, 12 potential pairs of oligonucleotide 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 ( $T_a=66^\circ\text{C}$ , 35 cycles) and P1F1R1TX-PCRs ( $T_a = 66^\circ\text{C}$ , 35 cycles), which actually were specific to the

**A**

GTTTCGCTCCCAGTTGATGTGGTTAAAACAGATTGTTTTATCATCCCTCAAATTAACA 60  
 CAGGTAAAATTTGCTCTAGGCAAATAATCGACAATAACTTTTTCTCATGATGACGTCAT 120  
 TCTTCATCCACGTTAAAATAAATGGTTCTCTTCTTAGCTTGCTCCATCTACCTTTGA 180  
 GTGGTTCATCACCCGAGTCAAAAATACTTATTAAAAACATTTGATTTTTGAGCAGCACGTTG 240  
 TTCCACTACCGCATACCGCGCCTTCTTTAAAGGTTTGGCAACAAGTTGTAGGAGTAACTA 300  
 GCTCCTACGGCTTTGTAAGCCAATGAAACTCGGTACGTAGAGCAGCAGACAAGTTCAAAT 360  
 CACATCCACCAGTTCAACTGACATTTTATTATAAAAAGTTGAAAAATAACAGAGCACAAT 420  
 AACGAGTACCATACCGAGAGACTGACGGATAACTCCACCTTATAATGCATCCATAACAAGC 480  
 AAAaCGCACGAAACTTTCTGGTCACGTGGTAATAAGAAGTAATTTTTTAAACAGTCTCA 540  
 CATTAAATGAATTTAGAAAGTGCCTATAAGTTACAACGAGTCATTGTATCGAGGTGTCTA 600  
 AGCCCTTCCCTACACACCGACAGTCAAGCTGGATTGAGTGTGAGGGACTCTTTgCAGTC 660  
 ACAAAGTCATTCAAGCGTTGGC 683

**B**

TGTTTCGCTCCGTAGTAGCTTATCGGTTTAAACAGGAGTACAAAGCATTCTTCTCACCCC 60  
 AGAACTGTGTAGACATAGAAATGCCCAAGGCCAACAGATACCTCTGAACCCGTAGTT 120  
 TTTCTGCCTCCCAAGTTATTTGCTAGACCTTTGAgTCGAATGGTGACATGAACATTAAG 180  
 TTCGAGCTCCGAAAATTTAAAACGAATGAATACCATGCACAATCAGAAGATGAgCAAAG 240  
 TTTGCAACGCAATTTGCAATTTGGAAAGAAACACAATGAAACTCCGATGACTAAACATG 300  
 CATCGCACCCACCGTCTCTGAAATATTCTATTGCTCATAGCTGATAAATTCACAACGCCG 360  
 TTA CTGATGCGTGGGAAAGTATCATTAAAGGCGTACTGACTTTCCCAAATGGATAGGCAA 420  
 CAACCTGATGGCGGTGGGAAAGTATCATTAAAGGCGTACTGACTTTCCCAAATGGATAGG 480  
 CAACAACCTGTGCTAACTTTGGAAAAGGTGAGTTTTGTTATTTGAATCCCAAAATCCCA 540  
 ATGTACAAAGTGTGACAGATATTA AAAAGTCGCATCGAATACAGCCCTTGAATCCGC 600  
 TCTGAACGGTAGTACCGGCGGCTACTCACGTGACCTGTTCAAGTACAGCCTGATGCAGGC 660  
 TGAGTTCTCGTCTATCGCATTGACTGTCACAATACATCCTTACACGCACACACCCGGGG 720  
 TATGGGTGTTGGACCGATGAACAGAAGGGTGGACATGGCAGGC GCTCACAGTGCAGGCC 780  
 ATATTCTGAGAAAGACCTGAAAATTCGAGGAAAGACCGGACACGGTCACTCGCCTCATT 800  
 CAATAACTGAGAAGAATAATCTTTGCAGGAGCGAAAC 877

**C**

GGGTTAAGCGGCCCTCGTACGCTGCAGCCCGGCCCATGGCCGCGGGATGTTTCGCTCC 60  
 TGT TTTAGTGTCTTGCCTTTCCTTTTAAATGTTTTTTTTTTTCAGTACTTTTCTCATGC 120  
 TGTTGCTGCTGTCATGTGACACGTGATTGTGTAGACAATTGACAATTGCTTTGTAGCTCG 180  
 TCAAATGTCCAAACATAAGAGGATAGTTAGTTTAAATCAACCTTCTGATACTGATTTAAA 240  
 ATTGACAGGATCTTCATAATTGTTGCTGCCAGTTCGATTA ACTCCTTTAACAGCGTAGT 300  
 GAAACTGTTATGCTCTGTTGTAGGAGCAATAGTTTGGCATCGAAATTCGGATTGGGAATG 360  
 AGAAATTTGAATCCGATTAAAGTTGGGGCTCGAGTATTGCCACAACCGAGTGCATGCTTT 420  
 GGCGGTTGGTGCATGAAACTTGATAGGGGGAGCTGGTACCAGGGGCCATTACAAAACCCG 480  
 R1TX ← → R2  
 TACCTTTTTTGAGATGCGCAGTCATCGTCATGGTACCGGTGATGATGTAAGACTTTAAGG 540  
 TGCTCGCTTATTGCAGTA<sub>g</sub>GTCTGAGTGCTTGAGGTCATCTCTGAAAGTTAGTATTTGGT 600  
 TTGTTGGATATTCCGCTCGAGAGATTGCAGTTTTAATTGGACAATTGTGATTTAATAA 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 and P1b 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 <sup>-1</sup>	18	6.65	
CaCO <sub>3</sub> g kg <sup>-1</sup>	150	137.28	
Total N g kg <sup>-1</sup>	0.78	0.28	
Available P mg kg <sup>-1</sup>	9.3	3.28	
Cation exchangeable capacity cmolc kg <sup>-1</sup>	32.0	27.5	
Exchangeable cations, cmolc kg <sup>-1</sup>	Ca <sup>+2</sup>	19.8	20.09
	Mg <sup>+2</sup>	4.5	3.04
	K <sup>+1</sup>	2.7	1.33
	Na <sup>+1</sup>	0.15	0.10
Microelements, mg kg <sup>-1</sup>	Fe <sup>+2</sup>	3.36	1.32
	Mn <sup>+2</sup>	1.74	1.17
	Zn <sup>+2</sup>	1.75	1.14
	Cu <sup>+2</sup>	0.78	0.69
	B	0.16	0.08
Electric conductivity dS m <sup>-1</sup>	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 amplify DNA 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 (Hosseini and Islami, 1998). Similarly, sometimes differential diagnosis is required to rule out other parasitic infections that resemble echinococcosis (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<sub>2</sub>-C production.

Soil	B	Microbial population									CO <sub>2</sub> -C production			
		Rate (kg da <sup>-1</sup> )	Bacteria			Fungi			Actinomycetes			CO <sub>2</sub> -C		
			SP	FP	HP	SP	FP	HP	SP	FP	HP	SP	FP	HP
0-30	0	25.70 <sup>b</sup>	37.75 <sup>b</sup>	29.61 <sup>ab</sup>	36.16 <sup>b</sup>	57.03 <sup>b</sup>	46.77 <sup>b</sup>	16.99 <sup>b</sup>	22.25 <sup>b</sup>	16.65 <sup>b</sup>	6.48 <sup>b</sup>	9.94 <sup>ab</sup>	7.78 <sup>b</sup>	
	1	31.42 <sup>ab</sup>	42.00 <sup>ab</sup>	30.36 <sup>ab</sup>	42.75 <sup>ab</sup>	67.30 <sup>ab</sup>	53.26 <sup>ab</sup>	17.33 <sup>ab</sup>	25.19 <sup>ab</sup>	19.85 <sup>ab</sup>	7.79 <sup>ab</sup>	11.52 <sup>ab</sup>	8.40 <sup>ab</sup>	
	3	34.67 <sup>a</sup>	47.50 <sup>a</sup>	37.60 <sup>a</sup>	46.91 <sup>a</sup>	72.64 <sup>a</sup>	61.25 <sup>a</sup>	21.00 <sup>a</sup>	31.03 <sup>a</sup>	24.19 <sup>a</sup>	8.57 <sup>a</sup>	12.67 <sup>a</sup>	10.20 <sup>a</sup>	
	6	25.37 <sup>ab</sup>	47.00 <sup>a</sup>	24.80 <sup>b</sup>	40.48 <sup>ab</sup>	72.00 <sup>a</sup>	59.12 <sup>a</sup>	17.66 <sup>ab</sup>	26.95 <sup>ab</sup>	20.46 <sup>ab</sup>	7.33 <sup>ab</sup>	12.55 <sup>a</sup>	8.26 <sup>ab</sup>	
	9	13.34 <sup>b</sup>	30.40 <sup>c</sup>	16.90 <sup>c</sup>	25.44 <sup>c</sup>	43.63 <sup>c</sup>	35.68 <sup>c</sup>	11.66 <sup>c</sup>	14.71 <sup>c</sup>	11.43 <sup>c</sup>	4.96 <sup>c</sup>	7.70 <sup>b</sup>	4.94 <sup>c</sup>	
Ave.		26.10 <sup>b</sup>	40.93 <sup>a</sup>	27.85 <sup>b</sup>	38.35 <sup>c</sup>	62.52 <sup>a</sup>	51.22 <sup>b</sup>	16.93 <sup>b</sup>	24.03 <sup>a</sup>	18.52 <sup>b</sup>	7.03 <sup>b</sup>	10.88 <sup>a</sup>	7.92 <sup>b</sup>	
30-60	0	13.20 <sup>b</sup>	19.27 <sup>b</sup>	15.03 <sup>ab</sup>	52.10 <sup>b</sup>	76.29 <sup>b</sup>	59.67 <sup>b</sup>	25.93 <sup>b</sup>	42.39 <sup>b</sup>	33.19 <sup>b</sup>	2.71 <sup>b</sup>	4.15 <sup>b</sup>	3.42 <sup>b</sup>	
	1	14.36 <sup>a</sup>	23.56 <sup>a</sup>	16.81 <sup>ab</sup>	60.14 <sup>ab</sup>	89.12 <sup>ab</sup>	63.98 <sup>ab</sup>	32.09 <sup>a</sup>	49.65 <sup>ab</sup>	36.81 <sup>ab</sup>	3.12 <sup>a</sup>	5.05 <sup>a</sup>	4.02 <sup>ab</sup>	
	3	15.27 <sup>a</sup>	24.15 <sup>a</sup>	21.05 <sup>a</sup>	65.21 <sup>a</sup>	95.80 <sup>a</sup>	79.70 <sup>a</sup>	35.79 <sup>a</sup>	55.07 <sup>a</sup>	43.36 <sup>a</sup>	3.67 <sup>a</sup>	5.60 <sup>a</sup>	5.75 <sup>a</sup>	
	6	14.00 <sup>a</sup>	24.00 <sup>a</sup>	19.79 <sup>a</sup>	57.37 <sup>ab</sup>	95.00 <sup>a</sup>	64.38 <sup>ab</sup>	29.93 <sup>ab</sup>	54.50 <sup>a</sup>	38.03 <sup>ab</sup>	3.23 <sup>a</sup>	5.21 <sup>a</sup>	3.92 <sup>ab</sup>	
	9	8.34 <sup>c</sup>	14.57 <sup>c</sup>	11.23 <sup>b</sup>	39.02 <sup>c</sup>	59.54 <sup>c</sup>	39.36 <sup>c</sup>	18.89 <sup>c</sup>	32.02 <sup>c</sup>	21.29 <sup>c</sup>	2.45 <sup>b</sup>	3.29 <sup>b</sup>	2.74 <sup>c</sup>	
Ave.		13.03 <sup>c</sup>	21.11 <sup>a</sup>	16.78 <sup>b</sup>	54.77 <sup>c</sup>	83.15 <sup>a</sup>	61.42 <sup>b</sup>	28.52 <sup>c</sup>	46.73 <sup>a</sup>	34.54 <sup>b</sup>	3.04 <sup>c</sup>	4.66 <sup>a</sup>	3.97 <sup>b</sup>	

SP, Sowing period; FP, flowering period; HP, harvest period. Bacteria 10<sup>6</sup> CFU g<sup>-1</sup> soil; Fungi 10<sup>4</sup> CFU g<sup>-1</sup> soil; Actinomycetes 10<sup>3</sup> CFU g<sup>-1</sup> soil, CO<sub>2</sub>-C mg C m<sup>-2</sup> h<sup>-1</sup>.

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 anthelmintics 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-molecular 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.

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