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Molecular typing of the fresh water snail *Lymnaea arabica*, the possible intermediate host of *Fasciola hepatica*, collected from Saudi Arabia, by RAPD-PCR

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ABSTRACT

Fasciolosis is caused by *Fasciola hepatica* with *Lymnaea* spp. as intermediate hosts. *Lymnaea arabica*, as a possible snail host collected from the Kingdom of Saudi Arabia, was investigated by Random Amplified Polymorphic DNA polymerase chain reaction (RAPD-PCR) technique. Polymorphic bands from *L. arabica* were sought for fingerprinting (typing) and identification of this species. The resultant electrophoretic patterns would be used as molecular bio-markers for these molluscas. This will help in the identification of the snails at a molecular level for the first time in the country that, in turn, will help in disease control. This technique was proved to be an accurate, appropriate, efficient and valuable alternative to traditional identification tools of *Lymnea spp.*

Keywords: *Lymnaea Arabica;* Saudi Arabia; RAPD-PCR, *Fasciola hepatica*,. Polymorphism

INTRODUCTION

Fascioliasis is a zoonotic disease that is caused by a trematode of species *Fasciola hepatica* (liver flukes) with about 20 million human cases around the world in its natural distribution. It is considered as emerging disease in several parts of the world, mainly in South America, Africa, and Asia. It is, also, now recognized as a concern in public health due to its high pathogenicity (GayoMera and Sierra, 2010, GayoCuervoRosadilla *et al.*, 2011). The infection rise in recent years has been attributed to the climate changes (EstebanBargues and Mas-Coma, 1998, Mas-Coma, 2005, FoxWhiteMcClean *et al.*, 2011). Animal fascioliasis, on the other hand, is also an important veterinary problem in regions where domestic animals raised. The economic impacts consist of chemotherapy costs, liver condemnation, loss of productive capacity (e.g., meat, wool and milk), and reduction in growth rate (Rapsch DahindenHeinzmann *et al.*, 2008, Hurtrez-Bousses Meunier Durand *et al.*, 2001).

The intermediate host for fascioliasis are the Lymnaeidae snails members (Kingdom: Animalia, Phylum: Mollusca, Class: Gastropoda, Order: Basommatophora, Family: Lymnaeidae). They are distributed all over the world (Brown, 1994, Kaplan, 2001). It has "right-handed" shells and prefers the shallow waters, where temperature is relatively higher (Grant, 2001). Snails become infected with the worm when the ciliated miracidium hatches from eggs and penetrates the soft part of snail body. When the minute cercaria (a motile phase of the parasite) emerge from the snail, attach to the vegetation, and eaten by the definitive host, the cycle is completed (Sorensen and Minchella, 2001).

Many *Lymnaea* species snails have been proposed in different areas in the world. These hosts include: *Lymnaea stagnalis, Lymnaea viatrix, Lymnaea*

neotropica, Lymnaea cubensis, Lymnaea columella, Lymnaea cousini, Lymnaea humilis, Lymnaea diaphana, Lymnaea occulta, Lymnaea auricularia, Lymnaea natalensis and *Lymnaea arabica* (CorreaEscobarDurand *et al.*, 2010). Due to the fact the genus *Lymnaea* is prevalent in different freshwater environments worldwide, the taxonomy and the resultant classification for its species has been always an issue of disagreement(Feulner and Green, 1999). This is due to the fact that the morphology of snails shells collected from parts of Arabian Peninsula, for example, is thinner and more high-spired than the average morphological characters for either *L. auricularia* or *L. natalensis* (the predominant species) collected from different parts of the Peninsula(Brown and Gallagher, 1985). As result of this controversial, the species *L. arabica* has been introduced for specimens collected from some parts of the Peninsula (Brown and Wright, 1980, Wright and Brown, 1980, Feulner and Green, 1999). Several classical studies have been conducting in Fasciola and Lymnaeid snails in Kingdom of Saudi Arabia (KSA). Fasciola was first reported in Saudi Arabia many years ago (Magzoub and Kasim, 1978, MagzoubKasim and Shawa, 1979). These reports mentioned a low infection rate in human and relatively high infection rates in domestic and wild animals (GhandourTahir and Shalaby, 1989, Sarwat and Al-Shaiby, 1993, Banaja and Ghandour, 1994, Al-Teimi, 1995, SaeedSattiKhamis *et al.*, 2000, Abou-Zinadah and Fouad, 2005, El-Mathal and Fouad, 2005, Sanad and Al-Megrin, 2005). The majority of studies listed above have generally been focused on defining species using morphological description. Seven species of snails, including *L. arabica* were collected from fifteen localities in Aseer Region in KSA. In this study, *Lymnaea palustris and L. arabica* were collected from 9 localities of them (Bin Dajem, 2009).Precise classification and identification of this snail is essential in the disease control. Shell color, anatomy of the reproductive system variation and shape are the traditional means that are used extensively for identification and classification of snails' species and genera, including *L. arabica*. These features, even extensively used, have been proved to be a problematic in many cases(Evans, 1989). To overcome problems arise when these phenotypic features used, genetic studies have been introduced. Numerous techniques have been used in an attempt to understand lymnaeid taxonomy. These include: enzyme electrophoresis, cytology, immunological studies, allozymes, RAPD-PCR analysis and DNA sequencing (Puslednik Ponder Dowton *et al.*, 2009).

According the available literature, all of the conducted studies were morphology-based studies and there is still much to learn about the disease components in KSA (the parasite, final host and the intermediate host), especially, at a molecular level.

The present work aimed to develop a DNA fingerprint for *L. arabica*, a possible intermediate host of *F. hepatica* in the country, using RAPD-PCR technique.

MATERIALS AND METHODS

Snails collection and maintenance and morphological identification

Saudi Arabian *Lymnaea arabica* snails were collected from freshwater bodies in the Asser province. Snails were kept in plastic trays; each tray contained 25 snails in 1.5 liters of dechlorinated water, and was supplied with fresh lettuce leaves for feeding (Becker and Lamprecht, 1977). Trays were covered with a perforated plastic cover to reduce evaporation of water and escape of the snails. Trays were maintained at room temperature (27–29ºC) and aquaria were cleaned weekly for the removal of feces and dead snails (Schneck and Fried, 2005). Snail specimens were killed and

fixed (Paraense, 1976). Fixed specimens were identified by means of comparative morphology of the shells according to Paraense and Deslandes (Paraense and Deslandes, 1958).

DNA extraction *and Lymnaea arabica* DNA amplification by RAPD-PCR

Genomic DNA extraction from non-exposed Saudi Arabian *L. arabica* was done using the DNeasy Blood & Tissue Kit from Qiagen, according to Vidigal et al. (VidigalCaldeiraSimpson *et al.*, 2000). Kit buffers were prepared and genomic DNA extracted according to the manufacturer's instructions, including RNase A (200 µg/ml) in the extraction buffer. Genomic DNA was extracted from the whole individual snails. Extracted genomic DNA was kept at -70°C until use. .*Lymnaea arabica* was genetically typed by RAPD-PCR, using nine 10-base oligonucleotide primers as described by Oliveira et al. (OliveiraDa SilvaManzano *et al.*, 2010a) and Dajem et al.,(Bin DajemIbrahimAl-Quraishy *et al.*, 2011), with some modifications. The procedure was performed using the Qiagen® Fast Cycling PCR Kit (Qiagen). Each reaction was carried out in a final volume of 25 µl, with the final reaction containing a 1× concentration of reaction buffer, 50 ng genomic DNA, 4.0 mM magnesium chloride, 2 units Taq DNA Polymerase, 0.4 mM dNTP mixture and 10 pM of each 10-base primer. Universally used primers were Primers I (Pharmacia), OPA-1 (GE Healthcare Limited, Amersham Place Little Chalfont, Buckinghamshire), and Gibco-BRL 3 (Gibco-BRL). In addition, 6 of our own unique primers were used as shown in Table 1. The cycling procedure was performed using a PTC 200 Peltier Thermal Cycler (MJ Research, USA) with the following program: predenaturation of DNA at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min, and a final extension of 2 min at 72°C

Table 1: Primer used in RAPD-PCR amplification of *L. arabica* genomic DNA

Primer name	Sequence (5' to 3')	PROBEDB_PUID
Saad-2	GTGCTACGTC	10554779
Osama-5	GACCATCGTC	10554777
Osama-6	CCGGCCTTCC	10554778
Essam-7	GATCGGACAC	10554774
Essam-8	GCTCGAACTT	10554775
Essam-9	CCACGCGCAA	10554776

Gel analysis

The PCR products were separated on a 1.3% agarose gel. The gel was photographed using a gel documentation system (Quantum-Capt, Viber Lourmat). Migration distance and the band sizes were calculated.

RESULTS

Morphological identification of snail shells

Fifteen specimens of the snails were collected from 9 localities in Aseer Region. They were killed and fixed..Its shell is dextral, slender with a pointed high spire. The shell measured from 4-10 mm(mean 6.9 mm) (Fig. 1). Snails were identified and classified according to the shape and the color of the shells. The snail's classification was confirmed by Prof. Thomas Kristensen (Danish Bilharziasis Laboratory, Denmark).

Fig. 1: Morphological characteristics of Saudi Arabian *Lymnaea arabica* snail shells collected from freshwater bodies within the Asser province.

Genomic DNA extraction and RAPD-PCR analysis

Genomic DNA extracted using the kit method resulted in a clear, distinct band without a contaminating smear (Fig. 2. A). Gel analysis of the RAPD-PCR products revealed many PCR bands (Fig. 2. B). Amplification using the P1 primer (Pharmacia) resulted in 12 different bands, ranging from 419 to 1894 bp. Amplification using the primer Saad-2 showed no bands, while amplification using the primer OPA-1 resulted in 11 different bands, ranging from 419 to 2369 bp. Amplification using the Gibco-BRL-3 or Osama-5 primer resulted in 1 band each, of 988 bp and 761 bp, respectively. Amplification using the primer Osama-6 resulted in 9 different bands, ranging from 423 to 2030 bp.

Amplification using the primer Essam-7 resulted in 8 different bands, ranging from 518 to 1912 bp; primer Essam-8 resulted in 6 different bands, ranging from 541 to 1912 bp; and primer Essam-9 resulted in 6 different bands, ranging from 344 to 1603 bp (Table 2).

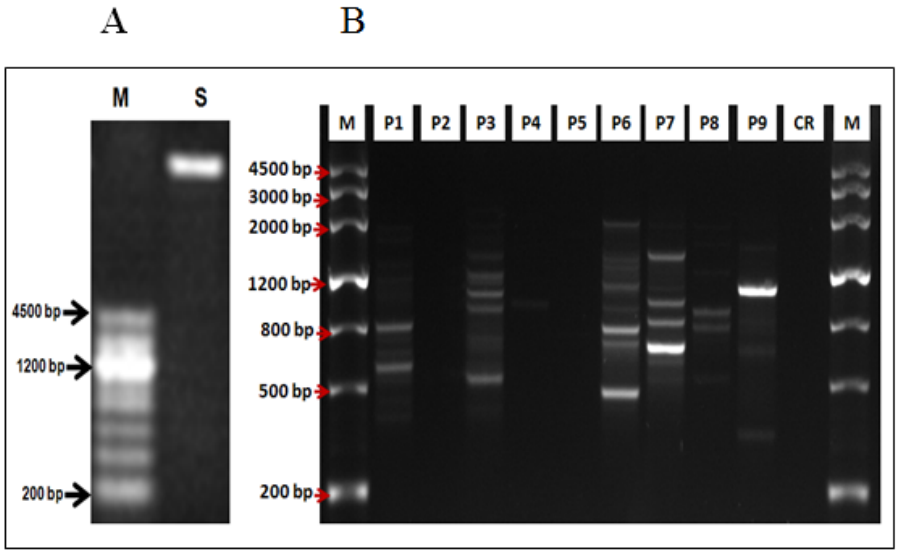

Fig. 2. A: Purified genomic DNA from a *Lymnaea arabica* snail.Lane M: GelPilot Wide Range Ladder; Lane S: *Lymnaea arabica* genomic DNA.

B: Electrophoretic pattern of amplified *Lymnaea arabica* genomic DNA using different RAPD-PCR primers. Lane M: GelPilot 50 bp ladder; Lanes P1–P9: different RAPD primers; and Lane CR: control reagent

Table 2: DNA fragments after RAPD-PCR amplification using different primers.

Primer#								
P1 Pharmacia	Saad-2	OPA-1	Gibco-BRL-3	Osama-5	Osama-6	Essam-7	Essam-8	Essam-9
RAPD-PCR products (kbP)								
1894	-	2369	988	761	2030	1912	1912	1603
1704	-	1842	-	-	1457	1553	1653	1108
1396	-	1473	-	-	1337	1098	1294	825
1200	-	1226	-	-	1149	1008	940	761
1037	-	1088	-	-	959	850	816	659
833	-	940	-	-	792	672	541	344
725	-	746	-	-	704	596	-	-
647	-	678	-	-	487	518	-	-
586	-	591	-	-	423	-	-	-
522	-	536	-	-	-	-	-	-
446	-	419	-	-	-	-	-	-
387	-	-	-	-	-	-	-	-

DISCUSSION

The aim of this study was to test the ability of RAPD-PCR method to identify and create the fingerprint of *L. arabica* using our own random primers as well as published primers. This molecular method was shown to be adequate technique and a good initial approach for distinguishing between closely related snails(Da SilvaSpadaSobral-Hamaguchi *et al.*, 2004). This methodology emphasizes our interest in determining the unique genetic code associated with the *L. arabica* strain. Amplification of genomic DNA extracted from *L. arabica* resulted in a fingerprint pattern unique to that snail. Historically, the lymnaeid shell features have been considered reliable characteristics and were generally the primary method used for species identification. Intra-species variation of shell shape is common throughout the Lymnaeidae and is thought to be a response to the relative transience of most freshwater habitats (Russell-Hunter, 1978). To overcome problems associated with phenotyping and anatomical variation classification of the shell, several genetic techniques have been utilized. Of these methods, DNA sequencing has been proved to be the most reliable tool in snail identification and classification.

Due to the important role of these species as intermediate hosts of many trematodes, a precise taxonomy of this snail is essential as the accurate classification of intermediate host will help in characterization of regions of epidemiological possibilities. This also will augment our understanding of the interaction between the correct snail host and the parasite.

In the current study, we analyzed the RAPD-PCR products using agarose gel electrophoresis as a rapid, reliable and inexpensive method for classification. Some researchers have used the same method (e.g.,(Oliveira et al., 2010a)), but others have used the alternative polyacryl amide gel electrophoresis method (Spatz Vidigal Silva *et al.*, 2000, Abdel-Hamid Rawi and Arafa, 2006, Oliveira Da Silva Zanotti-Magalhaes *et al.*, 2008, TeixeiraSouzaVidigal *et al.*, 2010).

The RAPD method has been originally used to detect SNPs and in taxonomy. it is a reliable, sensitive and reproducible assay(Oliveira et al., 2010a). This technique, however, has main drawback of lacking of reproducibility. This can be overcame, however, by the proper optimization of the technique (Atienzar and Jha, 2006).

This method has been proved to be useful as the resultant electrophoretic profile of *Biomphalaria arabica* that was collected from Saudi Arabia showed specific

polymorphic markers that can be used for taxonomy and identification (Bin Dajem et al., 2011). It has been shown also that the Genetic variabilities between strains of *Biomphalaria glabrata* that are susceptible and unsusceptible to *schistosoma* could be determined by this technique (OliveiraAhmadMaria *et al.*, 2010b). Such Molecular assays have been used to detect the parasite *F. hepatica* in its intermediate host and can be used as a valuable epidemiological tool for the purpose of snail infection monitoring methods (Kozak and Wedrychowicz, 2010). The tested primers in this study were adopted because they have not been used in prior studies for this snail. The results indicate that all of these primers tested identified polymorphic markers characteristic to *L. arabica*.

In conclusion, fingerprinting using RAPD-PCR will be an adequate tool to differentiate between closely related species of *Lymnaea* snails. For future research direction in this matter, we will sequence some of these specific bands and design specific primers adopted to classify this snail species.

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