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FIRST MOLECULAR IDENTIFICATION OF ADULT HETEROPHYESHETEROPHYES AND HETEROPHYESDISPAR (DIGENEA: HETEROPHYIDAE) FROM KUWAITI STRAY CATS USING ITS2 SEQUENCE

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ABSTRACT

In September 2015, adult specimens of the trematodesHeterophyesheterophyes and Heterophyesdispar were obtained from two stray cats captured near the fish market of Kuwait City. The rDNA ITS2sequencing and subsequent phylogenetic analysis with other heterophyids in the GenBank showed a close relationship with adult H. heterophyes, and Heterophyes sp.-small metacercariae from Sardinia in addition to adult Korean heterophyesnocens. While it is clustered separately from Kuwaiti Heterophyidcercariae obtained from Cerithideacingulate snail and the Indian heterophyid.Due to the close relationships between these trematodes, it suggests that the origin of the Kuwaiti adult H.heterophyes and H. dispar could be from the imported Mediterranean Sea fish and not the local one.Mullet (Mugilidae) is the most probable second intermediate host for both trematodes. This was the first molecular characterization of adult H. heterophyes and adult H. dispar from the Middle East and the first one in the natural definitive host.

KEYWORDS: *Heterophyesdispar, Heterophyesheterophyes, ITS2 rDNA, Kuwait*

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INTRODUCTION

Foodborne trematodiases cause two million life years lost to disability and death worldwide every year; People become infected by eating raw fish, crustaceans or vegetables that harbor the parasite larvae. These zoonoses are most prevalent in East Asia and South America (WHO, 2017). Fish can be infected by several trematode families, and only some of them include species pathogenic to humans, i.e.: Clinostomatidae, Echinostomatidae, Heterophyidae, Opisthorchiidae and Troglotrematidae (Waikagul&Thaenkham, 2014). In Kuwait, there were several records indicating the presence of heterophyid intestinal flukes at its various developmental stages (Abdul- Salam & Sreelatha, 1996, 1998; Abdul- Salam *et al*., 2000, 2004; Al- Kandari*et al*., 2000, 2015). In 1990, Abdul- Salam, and Baker found that the prevalence of *Heterophyesheterophyes* was 1.9% in cats, however it is strongly increased up to 15% in 2015 (El-Azazy*et al*., 2015).In addition, *Heterophyesdispar* was detected in the prevalence of 10.8% in the same year (El-Azazy*et al*., 2015). This rapid increase in the prevalence of *H. heterophyes* within fifteen years and the presence of *H. dispar* at such a high percentage along with the frequently reported human cases infected with *H. heterophyes* and *H. dispar* in many neighboring countries (Chai *et al*., 1986.; and Massoud*et al*., 1981) shows the actual need for further research on these parasites. In the past, morphological analysis was the only applicable technique of identification for heterophyid intestinal flukes. However, morphology identification alone might be insufficient for accurate species identification because of the

small size of the adult stage and taxonomic characteristics combined with the invalidity of those characteristics to high morphological similarities between closely related species, like homoplasy, phenotypic plasticity, a lack of conserved structures and a lack of distinctive morphological characteristics (Waikagul&Thaenkham, 2014).

Currently, because of these difficulties, molecular biology has been employed to detect parasites responsible for parasitic diseases. (Tavares *et al*., 2011). The Ribosomal internal transcribed spacer2 (ITS2) region is regarded as one of the candidate genetic markers. Ribosomal internal transcribed spacer region is remarked as one of the potential DNA barcodes because its possesses a number of variables characteristics, such as availability of conservedregions for designing universal primers, the case of its amplification, and sufficient variability to distinguish even closely related species (Yao *et al*., 2010).

The ITS2 region has been successfully used to genetically identify several heterophyidae intestinal flukes (Al-kandari*et al*., 2015; Chuboon*et al*., 2013; Gamit*et al*., 2016; Masala *et al*, 2016; Skov*et al*., 2009 andSripalwit*et al*., 2015). Therefore, this study aimed to identify the rDNA ITS2 sequences for adults *H. heterophyes* and adults *H. dispar* collected from stray cats in Kuwait and to use this identification to set a phylogenetic analysis of these trematodes with another heterophyid in the GenBank.

MATERIALS AND METHODS

Sample Collection

Two stray cats were captured near Downtown Kuwait fish market in September 2015 and taken to the lab. Cats were anesthetized using Rompun 2% intramuscular (1.5 ml/kg) and humanely killed according to the ethical standards for research by using intracardiac injection with T61 (Schering-Plough Intervet, Elkhorn, Nebraska, USA), 1-4ml according to the age and weight (El-Azazy*et al*., 2015). The intestine was removed and placed in separate trays, the mucosa was scraped, and the intestinal content was rinsed with saline and examined under a stereomicroscope for adult trematodes.

Some isolated parasites were stained with lactophenol cotton blue (Henedy and El-Azazy., 2013) and identified based on the morphological criteria described by Soulsby (1982) and Bray *et al*., (2008). The non-stained specimens were kept in 95% ethanol for subsequent molecular analysis.

DNAExtraction and PCRAmplification

After morphological identification, three specimens of each species were chosen for molecular analysis and washed with double-distilled water. DNA was extracted following the tissue protocol of genomic DNA Mini Kit (Geneaid, Teipei, Taiwan).

The rDNA ITS2 region was amplified using the primers OPHRT- F (CTC-GGC-TCG-TGT-GTC-GAT-GA) and OPHRT- R (GCA-TGC-ART-TCA-GCG-GGT-A) (Skov*et al*., 2009). The PCR reactions were conducted with 35 µl (25 µl Top Taq polymerase (Qiagen, Hilden, Germany) 0.2 µl for each primer, 5 µl of g DNA and 4.6 µl dd H_2O). The amplification consisted of an initial denaturation step at 95° C for 3 min followed by 35 cycles of 95° C for 30 sec, 50° C for 30 sec and 72° C for 45 sec, followed by a final extension of 8 min at 72° C. Products were resolved by electrophoresis on a 1.0% agarose gel and visualized with 0.5 mg/ml ethidium bromide. PCR products were purified using ethanol precipitation method according to the tissue protocol (QIAamp DNA Mini Kit, QIAGEN).

DNA Sequencing

The purified PCR products were sequenced using Big Dye Terminator chemistry with the same primers of the PCR amplification. The DNA sequencing reactions were electrophoresed on ABI's 3730XL DNA Analyzers (AIT biotech, Singapore). The obtained electropherograms were checked and edited using Bio Edit (Hall, 1999).The ITS2 sequences were aligned with Clustal X2(Thompson et al., 1997) and deposited in the GenBank database with the accession numbers KX431323-KX431328.

Phylogenetic Analysis

One obtained sequence from each species was aligned with those of other trematode species of the family Heterophyidae deposited in the GenBank, *Echinostomarevolutum* (accession no: LC224085) was used as an outgroup (Table 1). Phylogenetic tree analysis was conducted using maximum likelihood, performed using MEGA program version6 (Tamura *et al*., 2013). All ITS2 nucleotides were assembled in 1000 replications.

RESULTS

A total of 43 adult trematodes were recovered in the two stray cats, 27 of them were identified as *Heterophyesheterophyes*(Fig. 1) from one cat and 16 as *Heterophyesdispar*(Fig. 2) from the other cat. Three trematodes which were identified as *H. heterophyes* and included in the molecular analysis found to be genetically identical, and having the same number of bases (431), while the other three specimens which were identified as *H. dispar*, two were identical but one had one base difference at the position number 300 (G instead of A). By comparing these trematodes in the phylogenetic trees, we can find that all trematodes belong to the genus *Heterophyes* were clustered in one clade. In addition, *H. heterophyes* in this study form a monophyletic clade with *H. heterophyes* from Sardinia and both trematodes are a sister clade with Kuwaiti *H. dispar* (Fig.3).

DISCUSSIONS

H. heterophyes is a minute fluke that was discovered in an Egyptian child in 1851 by Bilharz (Schmidt & Roberts, 2005). The most characteristic feature of this fluke is that the genital sucker lies directly behind the ventral sucker and bears an incomplete circle of 70-80 small toothed spines (Soliman, 2006). *H. dispar* was first discovered in the intestine of dogs and cats in Egypt by Looss in 1902 (Motarjemi*et al*., 2014), It can be distinguished from *H. heterophyes* by the smaller sized genital sucker and the smaller number (22-33) of chitinous rodlets on the genital sucker (Chai & Lee, 2002), both of them are zoonotic trematodes which distributed in several countries, (Ashford &Crewe, 2003, Chai *et al*., 1986, Hung *et al*., 2013, Rifaat*et al*., 1980 and Yu& Mott, 1994).

The first intermediate host is brackish water snail and the second are fish species such as *Mugil*spp, *Liza* spp (Paperna& Overstreet, 1981), *Tilapia nilotica*, *Aphaniusfasciatus*, *Acanthogobius* sp. (Yu & Mott, 1994), *Flavimanus* sp. (Seo*et al*., 1981) *Chelonhaematocheilus* (Hung *et al*., 2013) and others (Abou- Aisha *et al*., 2008, Chai & Lee, 2002, Chai *et al*., 1986 and El-Sheikha& El-Shazly, 2008) in the case of *H. heterophyes*. Fishes such as *Mugil*sp(Paperna& Overstreet, 1981), *Epinephelusfasciatus*,*Lichia*sp, *Barchuscallipterus*, *Tilapia*sp (Hung *et al*., 2013), *So.vulgaris* and *Sc.aquilla* and others (Chai *et al*., 1986)were reported as second intermediate hosts in the case of *H.dispar*, while the definitive host is cats, dogs, foxes, wolves, (Chai *et al*., 2005) in addition to man (Yu &Mott, 1994) for both trematodes. The definitive host (e.g. Cats) becomes infected by ingesting undercooked or salted fish infected with metacercariae(CDC). Cats in this study

were captured near the fish market, where they feed on fish or fish offal.

Fish which arrive at the fish markets came through two routes, either local or imported; fish could become infected from the first intermediate host (snails). In Kuwait several reports indicate the presence of Heterophyidaecercariae in snails such as *Cerithideacingulata*,*Clypeomorusbifasciataus,* and *Cerithiumscabridum* (Abdul-Salam& Al-Khedery, 1992), (Abdul-Salam & Sreelatha, 1993,1998), (Al-Kandari*et al*., 2000)and (Abdul-Salam *et al*., 2004), but currently no studies showing the presence of *H. heterophyes* or *H. dispar*metacercariae in local fish. Although Al-Kandari*et al.*, (2013) sequenced trematodes from Kuwait and identified them as Heterophyidaesp, when comparing such sequences with those obtained in this study, there is a significant distance between the clades. (Fig.3). Therefore, we could suggest that they are not belonging to the same genus or species, and the source of the recent trematodes could be from imported fish.

In Sardinia, three adults of *H. heterophyes*, obtained from an experimentally infected hamster with metacercariae found in *M. cephalus*, were used to obtain the molecular sequences of ITS2 and 28S regions. As shown before, *H. heterophyes* sequences in this study clustered in a sister clade with the Sardinian *H. heterophyes*, this result suggests that *H.heterophyes*might have arrived in Kuwait through imports. *H.dispar* in this study is not performing a monophyletic clade with the Sardinians one although they are grouped together; this may be due to the variation in the 2nd intermediate host which could be from Mugilidae other than *C. labrosus* and *L.ramada*.

In 2015 Kuwait imported about 10,077 tons of different types of fish, compared with 3,860 tons of local fish (Annual Bulletin, fisheries statistics- central statistical Bureau. the State of Kuwait, 2015). Fish are imported from many countries such as Saudi Arabia, Oman, Iran, India, Pakistan, Egypt, Turkey and others from Far East countries (Director of the fishery section at Kuwait Municipality Mr. Mohammad Al Failakawi pers comm).

In Saudi Arabia, *H. heterophyes*metacercariae were detected in one fish species *Mugilcephalus*(Khalil *et al*., 2014). While adult *H. heterophyes* were found in people and animals in Iran (Massoud*et al*., 1981), on the other hand, adults *H. dispar* were detected in cats of Madras in India (Rajavelu& Raja, 1988), but still, the source of infection in both Iran and India is unknown. In Pakistan; *H. heterophyes*metacercariae were present in some fish species which are not present in the Kuwaiti markets (Marcus *et al*., 2012).

Kuwait imports *Mugil cephalus* (Bori) and *Oreochromis niloticus* (Bolti) from Egypt, *Dicentrarchuslabrax* (European seabass), *Sparus aurata* (gilthead seabream) and Agyrosomus*regius* (meager) from Turkey.(Husain Al Sayegh, PAAF- Fisheries laboratory head pers comm).Egypt, Turkey,and Sardinia all are located in the Mediterranean Sea region.

Currently, there is no indication that the imported Turkish fish carry *H. heterophyes* or *H. dispar*metacercariae although it was found in other fish species (Öktener*et al*., 2010). However, several studies mentioned the occurrence of both heterophyids in both Egyptian fishes (Fahmy&Selim, 1959), (El- Shazly*et al*., 2007), (Abou-Eisha*et al*., 2008), (Berger, 2010). Suggesting the migration of these parasites originated from Egyptian imports. Furthermore; the snail *Pirenellaconica*was reported as a first intermediate host for both *H. heterophyes* (Taraschewski&Paperna, 1981) and *H.dispar* (Hung *et al*., 2013)in Egypt from the Mediterranean Sea (Taraschewski&Paperna, 1981).

Transmission of these trematodes occurs by consuming raw or newly salted or pickled fish. *H. heterophyes* infection is common in Egypt, where the pickled mullet (*Mugil cephalus*) is traditionally eaten at the feast of Sham- al Nessim (Woo, 2006). In1933, Khalil reported that encysted metacercaiae of *H. heterophyes* could remain in salted fish (locally known as Fessikh) and remain viable for at least week.

The human infections in Kuwait can occur through many pathways. In 2014 there were more than 2.4 million expatriates from more than fourteen nationalities living in Kuwait, of which, approximately half a million of them are Egyptians (Gulfnews.com).

Kuwaitis also have other food habits which can cause infection, grilling mullet without cleaning it, and sucking the head including the gills in addition to eating the flesh with the entire viscera, grilling fish by this way makes fish tender, oily and tasty according to local fish lovers. Grilling for 5 and 10 minutes was not sufficient to destroy all encysted metacercariae in fish muscles (Abou- Aisha *et al*., 2008). In mullet kept at 50 and 100°C respectively, the parasite lived for 180 and 10min (Hamad & Elias, 1970).

Furthermore; El-Azazy*et al*., 2015 mentioned that expatriate laborers have been observed cleaning fish then disposing of the offal in the garbage to be accessed by stray cats which are a good indicator of FZTs in the environment of Kuwait. The Ecology Global Network estimates that there are about 600 million small cats in the world. This includes pets, strays, homeless and feral cats. The wild cats alone number about 100 million. Those cats can be an excellent transmitter of FZT.

In Kuwait, most people will capture stray cats in the neighborhood and release them near the fish markets where they can find a frequent supply of fish and/or fish offal, this method can increase the concentration and types of trematodes that can be transmitted to those cats.

CONCLUSIONS

The results of the current study give a molecular data of adults *H. heterophyes*and *H. dispar* using ITS2 rDNA region and shows a close relationship between *H. heterophyes* from Kuwait and that from Sardinia and gives us an approximate answer about the source of *H. heterophyes* and *H.dispar*trematodes which could be the imported Egyptian fishes,(Mugilidae) especially *M.cephalus*.Further investigations should be done to examine all types of fishes that are present in the Kuwaiti fish markets for the detection of fish-borne trematodes.

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Competing interest: The author declares that he has no competing interest.

Declarations of interest: none.

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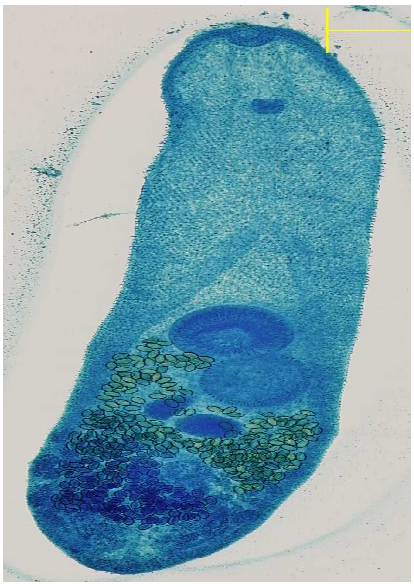

Figure 1: *Heterophyesheterophyes*

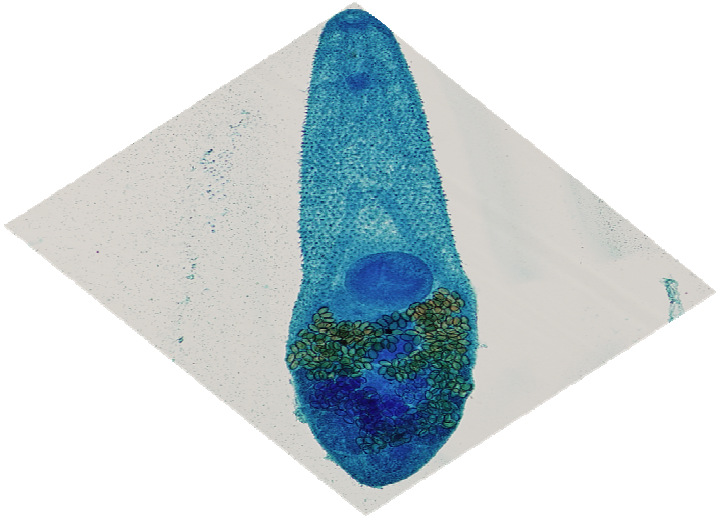

Figure 2: *Heterophyesdispar*

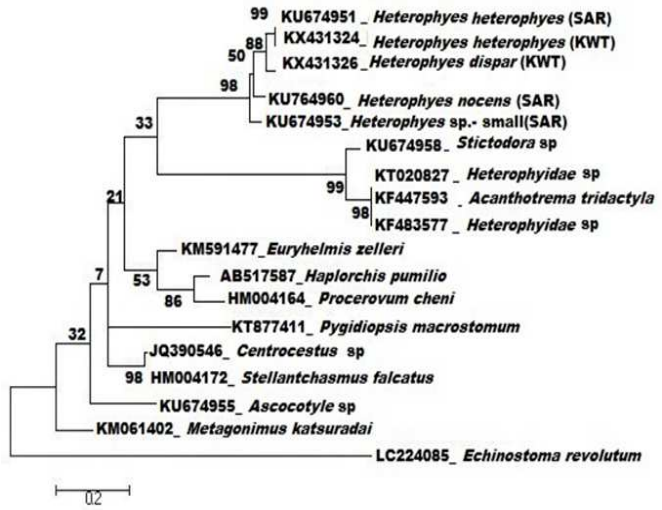

Figure 3:Maximum likelihood tree showing the phylogenetic relationships between the obtained *H. heterophyes* and *H. dispar* sequences and other trematode sequences from the GenBank based on ITS2analysis

Note: (KWT) KUWAIT. (SAR) SARDINIA

Table 1: Trematodes used for Phylogenetic Analysis with their Respective Genbank Accession Numbers

Trematode	Host	Locality	Accession Number
Acanthotrematridactyla	*Cerithidea cingulate*	Kuwait	KF447593
*Ascocotyle*sp	*Chelonlabrosus*	Sardinia	KU674955
*Centrocestus*sp	*Melanoidestuberculata*	Iran	JQ390546
Echinostomarevolutum	*Anas platyrhynchos domesticus*	Bangladesh	LC224085
Euryhelmiszelleri	*Bythinellaaustriaca*	Slovakia	KM594177
Haplorchispumilio	*Homo sapience*	Viet Nam	AB517587
Heterophyesdispar	Stray cats *(Feliscatus)*	Kuwait	KX431328
Heterophyesheterophyes	Adult: *Hamster*	Sardinia(Western Mediterranean Sea)	KU674951
Heterophyesheterophyes(This study)	Stray cats *(Feliscatus)*	Kuwait	KX431324

Table 1: Contd.,			
**Heterophyes*sp (small)	*Liza ramada*	Sardinia(Western Mediterranean Sea)	KU674953
Heterophyesnocens	Adult: Domestic cat	Sardinia(Western Mediterranean Sea)	KU674960
*Heterophyidae*sp		India	KT020829
Metagonimuskatsuradai	*Tanakialimbata*/lab host*: Mesocricetus auratus*	Thailand	KM061402
Procerovumcheni	*Anabas testudineus*/lab host:*Mesocricetus auratus*	Thailand	HM004164
Pygidiopsismacrostomum	*Poecilia vivipara*	Brazil	KT877411
Stellantchasmusfalcatus	Adult trematodes	Viet Nam	HM004172
Stictodorasp	*Liza saliens*	Sardinia(Western Mediterranean Sea)	KU674958