DOI : 10.35124/bca.2019.19.2.4155

Biochem. Cell. Arch. Vol. 19, No. 2, pp. 4155-4161, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

MOLECULAR STUDY OF HYDATID CYSTS IN HUMAN, SHEEP AND DONKEYS AT BASRA CITY, SOUTHERN IRAQ

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(Received 29 March 2019, Revised 26 June 2019, Accepted 10 July 2019)

ABSTRACT : Hydatid disease or Echinoccosis is one of the serious public health problems. This study designed for molecular investigation of Hydatid cysts in different hosts animals (Sheep, donkey) and human in Basra city, as well as molecular characterization by PCR technique to determine the specific gene for human (*ND1, COI, CO1*), sheep (*G6-7, sh4-1, COI*) and donkey (*COI*, *CO1*). Then, gene sequencing to recognize the intraspecific variation of *Echincoccus* in relation to the host. In the current study, 818 sheep were examined and the number of sheep infected with hydatid cysts was 271. Donkey samples (10) were brought to the animal field of Veterinary Medicine at the University of Basra and the number of infected with hydatid cysts was two in the period from September 2017 to March 2018. The hydatid cysts were collected from the human after surgery at Al-Sadr Teaching Hospital where 21. The results were higher in female (15) than male (6) in the same period.

Key words : Molecular study of hydatid cysts, Basra, southern Iraq.

INTRODUCTION

Hydatid disease or *Echinococcosis* infects herbivorous animals and human caused by *Echinococcus* spp. tapeworms. A cestode parasite usually a small tapeworm adult found in intestine of carnivores animals. There are two species of cestode affecting the human population, *Echinococcus granulosus* and *Echinococcus multilocularis* casing Cystic *Echinococcosis* (CE) and Alveolar Echinococcosis (AE) respectively, ordinarily seen in visceral organs like: liver, spleen and lung (Gottstein, 2003). This disease has serious impacts on human and animal health (Snabel *et al*, 2009) and possess a significant economic and public health problem in many parts of the world especially in rural areas where dogs and livestock are raised together (Sikó *et al*, 2011; Groeneveld *et al*, 2010). Echinoccosis is one of the serious public health problems in Iraq (Mohamad *et al*, 2008). It has been found that a high prevalence of hydatid cysts was in sheep (14.75) and a prevalence of hydatid cysts according to area of the study was 1.5%, 5.9% and 13.7% in North, Middle and South of Iraq, respectively (Mohamad *et al*, 2008).

The clinical diagnosis of (CE) in humans and animals were difficult because the disease without symptoms and the morbid recognition of the causative species was difficult in the cases of irregular forms (Eckert and Depalazes, 2004). *E. granulosus* showed a wide intraspecific difference in correlation to host specificity, epidemiology, morphology, developmental biology, biochemistry, physiology, biochemistry, and genetics (Thompson and Lymbery, 1998). For species identification, Nakao *et al* (2010) identified *Echinococcus* spp. by using molecular diagnosis, in this time, clinical samples taken at biopsy are subjected to PCR, and the amplified the fragments of mitochondrial and nuclear DNA are subsequently sequenced and determined strains.

MATERIALS AND METHODS

A total number of 818 sheep were examined and the number of sheep infected with hydatid cysts was 271. Donkey samples (10) were brought to the animal field of Veterinary Medicine at the University of Basra and the number of infected with hydatid cysts was 2 in the period from September 2017 to March 2018. In animals, determination of the age and sex of samples infected, determination of infected organs (lung, liver, etc.) and calculation the number of cysts in infected organs was done. Type of infection divided a according to the number of cysts into light infection (1-10 cysts) and severe infection (more than 10 cysts). Detected conducted of the prevalence of Hydatid cysts in slaughtered sheep in Basra (licensed abattoir) revised. The organs of slaughtered sheep were examined and the total number of examination was 818 infection in organs (liver, lung).

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In human, hydatid cysts from samples were collected from the patients who were recognized by surgery as having hydatid cysts inside body organs and done in their operation at Al-Sader Teaching Hospital. Human was examined during the period between September 2017 to March 2018. The study was conducted to detect the prevalence of Hydatid cysts in the Al-Sader Teaching Hospital in Basra province, The surgery was done to the patients after that the isolated cysts were taken and put in clean container with 70% ethyl alcohol and transported to the laboratory of Parasitology at College of Veterinary Medicine in Basra University. Some information also was taken, like: sex, organ and number of cysts in infected organ geographic location.

DNA extraction

From the germinal layer and liquid, DNA was extracted by using Wizard ® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions. The DNA was stored at -20ºC. To know the amount of DNA in sample after extraction making Nano drop and recorded the concentrate of DNA by spectrophotometry at 260 nm and 280 nm.

PCR ampliûcation of gene fragment

Primers was used for the amplification hydatid cyst of the following animals is shown in Table 1. In the current study, 6 primers used to determine the species of *Echinococcus*.

DNA of hydatid cyst was amplified by using PCR technique (GoTaq Hot Start Green Master Mix, Promega) depending the manufactures directive. Six pairs of primers were used to determine the species *Echinococcus*.

Typical PCR conditions are as follows: initial denaturation for 5min at 95°C. then 35 cycles of 95°C for 30sec, 56°C for 30sec, 72°C for 30sec. Then held the reaction at 72°C for 10min, and finally cooled down 4°C for 5min. The PCR product was then detected on agarose gel stained with ethidium bromide. While, the amount of PCR reaction which used in the cycling is shown in Table 2.

DNA sequencing and sequences analysis

The product of PCR was sent to Macrogene (Korea) company, for sequencing. Then the sequences were emended and aligned by using Parbi-Doua and NCBI BLAST programs. Compare the results with data obtained from Gene Bank published ExPASY program which is available at the NCBI online. Phylogenetic analysis was performed by using the NCBI program.

RESULTS

In the current study, 818 sheep were examined and the number of sheep infected with hydatid cysts was 271 (Fig. 1a and b). While, the number of donkeys infected with hydatid cysts was two. In human, the number of infected with hydatid cysts was 21, the female was higher infected in hydatid cysts (15) than (6) male in the same period. The current study showed that the rate of infection in sheep was (36.15%) in the licensed abattoirs. However, the rate of infection in donkeys was (28.5%). The total intensity of infection in human was between

Table 1 : The oligonucleotides used in this study.

Primer	Primer sequence (5' → 3' end)	Sources	Annealing temperature	Product size
ND1	GTTTTTGGGTTAGTCTCTGG	Sanchez *et al* (2012)	58 ºC	800bp
	ATCATAACGAACACGTGG			
CO1	TTTTTTGGGCATCCTGAGGTTTAT	Bowles *et al* (1992)	55 ºC	446bp
	TAAAGAAAGAACATAATGAAAATG			
COI	TTGAATTTGCCACGTTTGAATGC	Pour *et al* (2011)	56 ºC	792bp
	GAACCTAACGACATAACATAATGA			
CO1	TTTTTTGGCCATCCTGAGGTTTAT	Bowles *et al* (1992)	56ºC	446bp
	TAAAGAAAGAACATAATGAAAATG			
sh1-4	GTTATAAGAGGCCTCTCCGTGTTGTGG	Hosseinzadeh *et al* (2012)	56ºC	295bp
	CGTACGATTAGTTTCACAATATACATAT			
G6-7	TGGGGTAGTTACAATAGTTATTC	Hosseinzadeh *et al* (2012)	56ºC	234bp
	CATAATCAAATGGAGTACGATTA			

Table 2 : Amount of PCR reaction.

Substance	1 reaction	2 reaction
Master mix	12.5 ml	25 ml
Forward primer	1 ml	2 ml
Reverse primer	1 ml	2 ml
Template DNA	3 ml	6 ml
ddH_2O	7.5 ml	15 ml
Total reaction	25 ml	50 ml

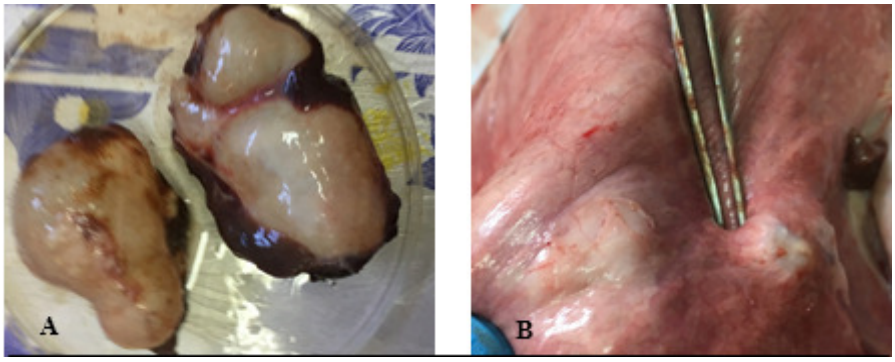

Fig. 1 : Sheep infected by hydatid cyst (A) in liver and (B) in lung.

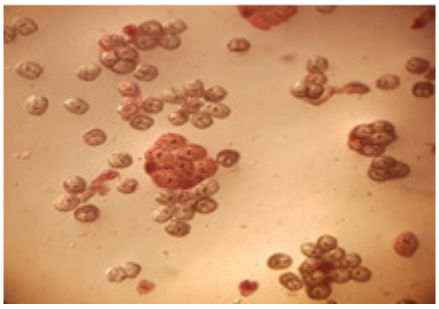

Fig. 2 : A live and dead protoscolices isolated from sheep.

done to detect the species of *Echinococcus* and identify the specific gene and species in Iraq, detect presence of six genes in (Sheep, Human and Donkey), including the sheep strain *G6-7* (234bp), *sh4-1* (295bp), *COI* (792pb), human strain *CO1* (446bp), *ND1* (800bp) and donkey strain *CO1* (446bp), *COI* (792bp). DNA was extracted using Wizard® DNA Purification Kit for PCR technique.

Fig. 3 showing the results of PCR for primers (*G6-7*, *sh4-1*, *COI*) in sheep samples, as well as Fig. 4

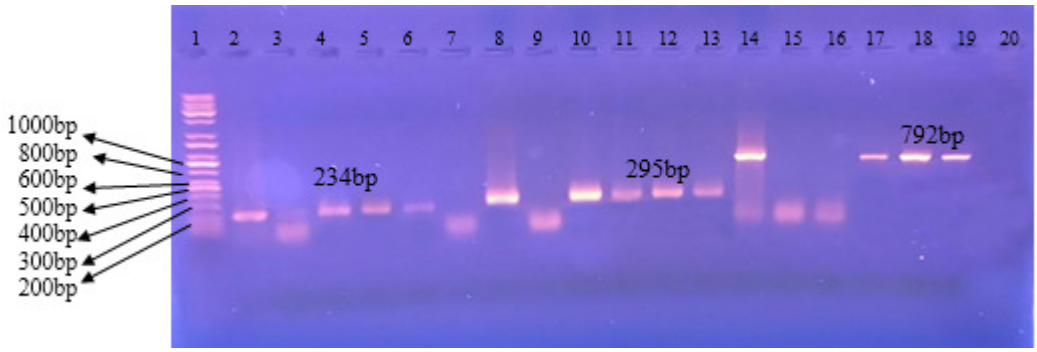

Fig. 3 : Results of PCR for primers (*G6-7, sh4-1, COI*) for sheep samples, lane (1) is the ladder, lanes (2,4,5,6) for *G6-7*, lanes (8,10,11,12,13) for *sh4-1*, lanes (14,17,18,19) for *COI*.

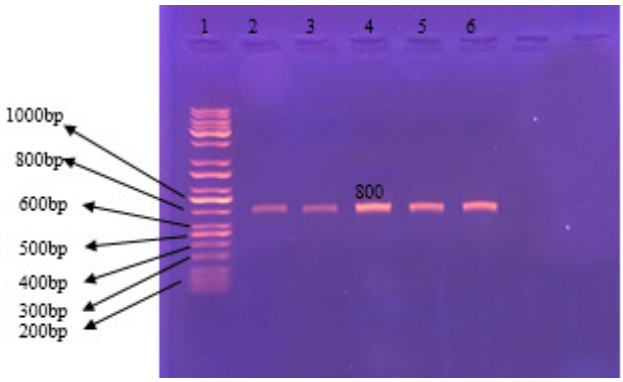

Fig. 4 : Results of PCR for primer (*ND1*) for sheep samples, lane (1) is the ladder, lanes (2,3,4,5,6) for *ND1* gene.

(4.43 and 4.4) males and females, respectively. In this study, the protoscolices collected from seven females sheep, the live protoscolices stained with green and dead with red color (Fig. 2).

In molecular study, determination the species of *Echionococcus* and limited presence group of genes was showing the results of PCR amplification for primer (*ND1*) for sheep samples. Fig. 5 appeared the results of PCR for primer (*CO1*) for sheep samples. In human, the results of PCR amplification for primer (*CO1*) showing in Fig. 6. While, Fig. 7 showing the results of PCR amplification for primers (*COI*) for donkey samples.

The results of the sequencing analysis of *G6-7* showed that 96% with Estonian isolation, *sh4-1* showed 99% with Iranian isolation. While, the *COI* gene showed 100%, the *ND1* gene showed (99%) and *COI* gene to determine *E. equines* showed (100%). Finally, *CO1* results showed 100% with Turkish isolation.

Sequencing

Sequence was done to determine the Iraqi strains, PCR product of *COI, G6-7, sh4-1, ND1* and *CO1* genes of *Echinococcus* spp., showed the identity of (*COI, G6-7, sh4-1*) genes and origin of isolate for *Echinococcus granulosus* in sheep (Table 3). Table 4 represented the identity of (*CO1, ND1*) genes and origin of isolate For

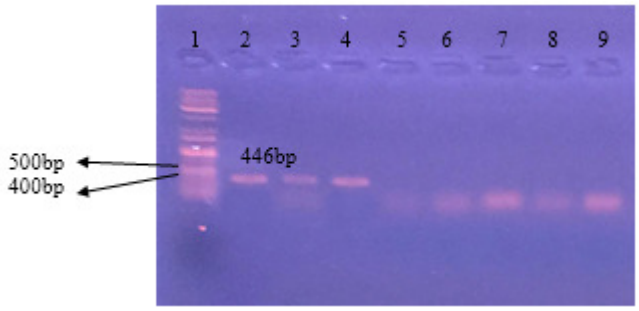

Fig. 5 : Results of PCR for primer (*CO1*) for sheep samples, lane (1) is the ladder, lanes (2,3,4) *CO1* gene.

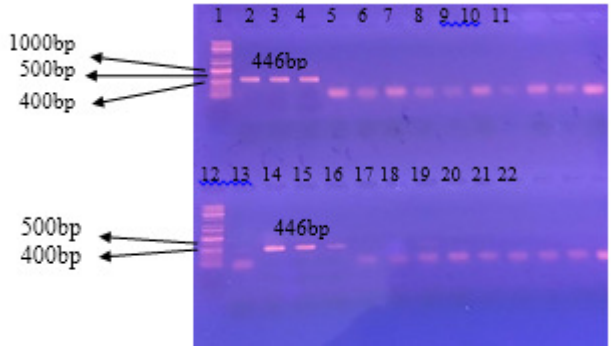

Fig. 6 : Results of PCR for primer (*CO1*) for human samples, lanes (1,12) is the ladders, lanes (2,3,4,14,15,16) *CO1* genes.

Table 3 : The identity and origin of isolate for *Echinococcus granulosus* in sheep for *COI*, *G6-7*, *sh4-1* genes.

Study sample	Compatible NCBI copy	Identity
G6-7	KX039965.1	97% with Estonia isolate
sh4-1	HM563031.1	99% with Iran isolate
COI	MG672293.1	99% with Estonia isolate
COI	MF281540.1	99% with China isolate

Table 4 : The identity and origin of isolate for *Echinococcus granulosus* in human and sheep for *CO1, ND1* genes.

Study sample	Compatible NCBI copy	Identity
CO1	MH010310.1	100% with Iran isolate
CO1	FJ608748.1	99% with Italy isolate
CO1	MF544127.1	100% with Turkey isolate
ND1	MG672293.1	100% with Estonia isolate

DISCUSSION

The present study showed that the total number of sheep and donkey infected with hydatid cysts was 271 and 2, respectively. The total percentage of infection in the study was 36.51. This finding is in agreement with previous studies (Umur, 2003 and Getawa *et al*, 2010). While, the prevalence was low compared with other studies around the world, in Morocco was 10.5%, in Kenya was 3.6% (Njoroge *et al*, 2002), in Iran was 15.5% (Hossainzadeh *et al*, 2012). In Libya, the percentage of sheep infected was (8.7%) (Al-Khalidi, 1998). In north and middle of Iraq, the prevalence of sheep infected was 1.5% and 5.9%, respectively (Mohamad *et al*, 2008 and Thweni and Yassen, 2015). The difference in prevalence reported by these studies might be accounted on the grounds of contrastive management practices, natural resistance, drug treatment and nutrition.

In human, the current study showed that the total number of human infected with hydatid cysts was 21 in both males and females, the higher infection recorded by Khalf *et al* (2014). In Baghdad, infection with hydatid cysts was 60 and in Hilla and Najaf hospitals were 61 (Al-Yasari *et al*, 2013). The difference between the results

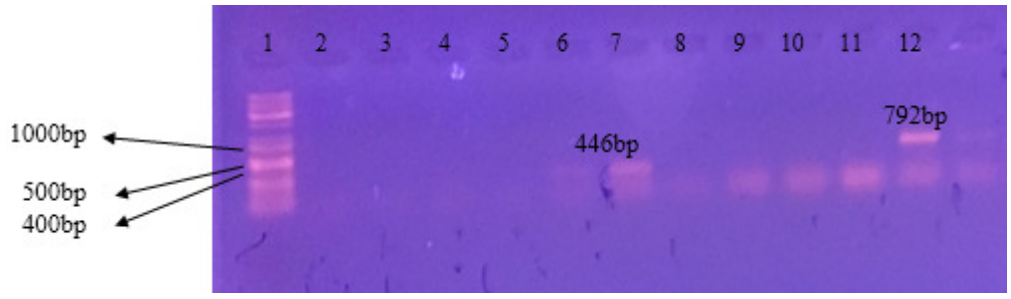

Fig. 7 : Results of PCR for primers (*COI* and *CO1*), lane (1) is the ladder, lane (12) *COI* gene and lanes (6,7) *CO1* gene for donkey samples.

Echinococcus granulosus in human and sheep. While, Table 5 showed the identity of (*COI, CO1*) genes and origin of isolate for *Echinococcus equines* in donkey.

Recorded in GenBank

In the present study, recorded some of gene in the GenBank, Table 6 showed the result of accession.

Phylogenetic analysis

Phylogenetic trees were generated by using NCBI program (Figs. 8, 9). The phylogenetic tree of gene *G6-7* for *E. granulosus* are shown in Figure 8, rooted neighbor joining phylogenetic tree showing the distribution and phylogenetic relationships between *E. granulosus* in Iraq and other countries.

Phylogenetic tree of gene *sh4-1* for *E. granulosus* are shown in Fig. 9, rooted neighbor joining phylogenetic tree showing the distribution and phylogenetic relationships between *E. granulosus* in Iraq and other countries.

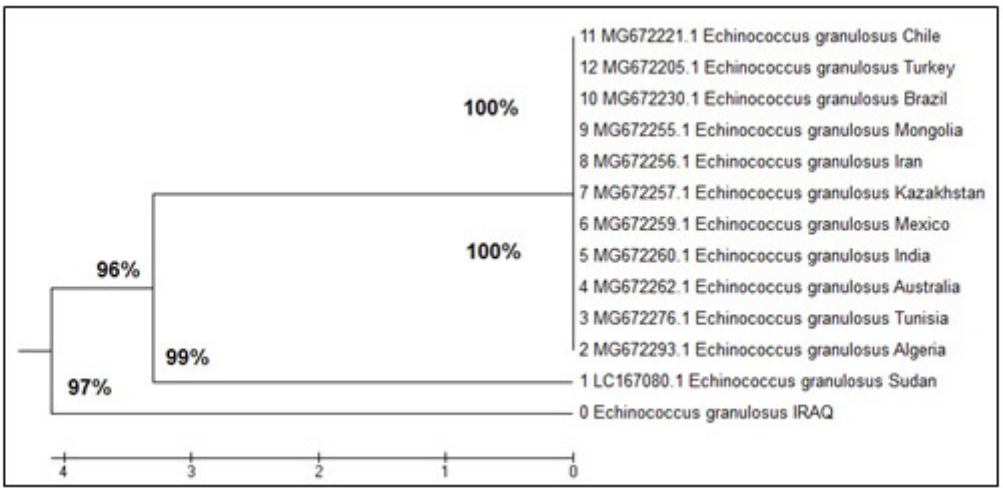

Fig. 8 : Phylogenetic tree of gene *G6-7* for *E. granulosus.*

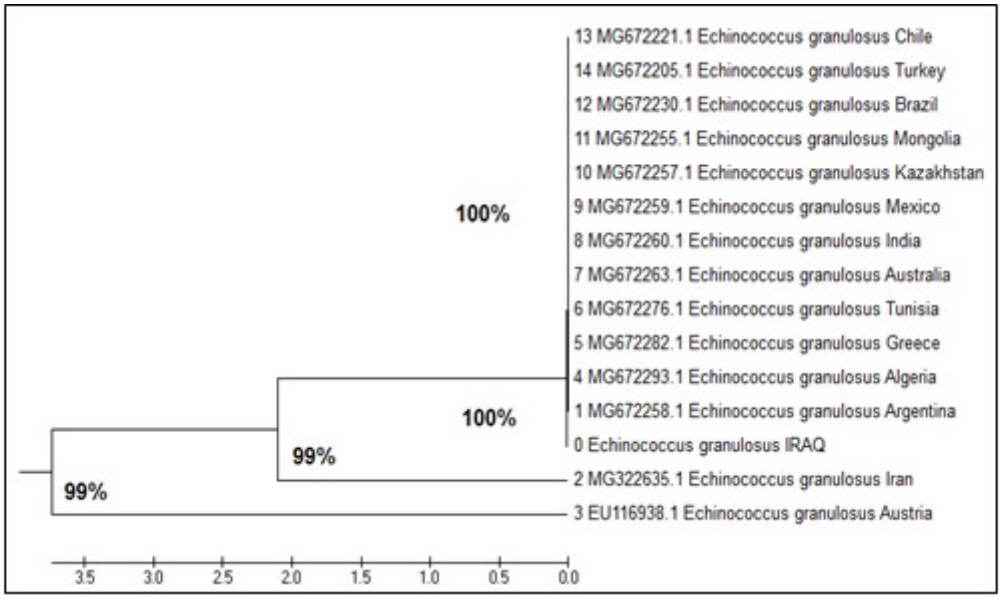

Fig. 9 : Phylogenetic tree of gene *sh4-1* for *E. granulosus*.

Table 5 : The identity and origin of isolate for *Echinococcus equines* in donkey for *COI, CO1* genes.

Study sample	Compatible NCBI copy	Identity
CO1	KY766905.1	100% with Estonia isolate
COI	KY766905.1	100% with Estonia isolate

Table 6 : The accession number of gene in GenBank.

No.	Type of gene	Accession number
1	*CO1*	BSeq#1
2	*CO1*	MK532394
3	*CO1*	BSeq#1
4	*ND1*	BSeq#1
5	*ND1*	MK532395
6	*ND1*	seq1
7	*CO1*	BSeq#1
8	*CO1*	BSeq#1

of this study and other studies because many reasons like the time of collecting samples were short, different seasons, the health conditions were different in Iraqi provinces.

The results of this study showed that hydatidosis is highly spreading disease in sheep in Basra city. Greater efforts are needed to control the transmission of hydatid cysts from abattoirs by the proper disposal of infected offal, especially of sheep to reduce the transmission of cysts from abattoirs to potential hosts in this region. Now, veterinary authority should apply a control program to control this disease through better standardization abattoirs system, increase knowledge of farmers toward hydatid cysts and eliminated stray dogs from Basra city (Mutar *et al*, 2017).

In molecular diagnosis of *Echinococcus* spp., the results of genes for sheep strain (*G6-7*, *sh4-1*, *COI*)

agreed with Hosseinzadeh *et al* (2012), who extracted DNA and used *G7-6* and *sh4-1* genes to detect *E. granulosus* in sheep. The results of amplification of these genes were (234pb) and (294pb) for sheep strain, whereas *COI* the aligned (792bp) sequence matrix of partial *cox1* gene contained 124 variable sites (Junying *et al*, 2012). This gene was used to determine species identification of *Echinococcus* (Pour *et al*, 2011).

ND1, CO1 genes were used for human strain, but in this study, three of extracted DNA showed positive results for *ND1* and 6 for *CO1* in the sheep. This finding might be the stray dogs. Stray dogs are one of the important causes for distribution of human strain in sheep because the stray dogs are infected by multi infection of *E. granulosus* and *E. multiaricularis* at the same time. These organism transform information between each other, throw out by defecation and the sheep become then infected by feces. The other reason is that the slaughtered animals in Basra city may come from other governorates, which may transmit this infection. The final reason is related to foxes or jackal or any other immigrant animals because border open with other countries and other governorates which lead to transforming this infection to Basra city.

The results of donkey samples for genes *CO1* is in agreement with Blutke *et al* (2010). In the present study, *COI* gene was used to determine *E. equnis*. The results of gene *CO1* agreed with Hama *et al* (2012), Baraak (2014) and AL-Nakeeb *et al* (2015) in Iraq. The results of the present study are consistent with studies in different parts of the world showing that the sheep strain represents the most important pattern responsible for human injury and a wide range of intermediate hosts (Busi *et al*, 2007; Andresiuk *et al*, 2009; Guoa *et al*, 2011). The results of Pezeshki *et al* (2012) in Iran reported that sheep strain is more prevalent in humans, sheep and goats. Utuk *et al* (2008) explained that the sheep strain is the dominant pattern in humans, cows, sheep, goats, and camels. *CO1* is a partial gene, three of human extracted DNA showed positive results for this gene because of multi infection.

In the present study, the results of sequencing showed that *G6-7* gene was a (96%) identification with Estonia isolate which was recorded in GenBank in accession number (KX039965.1), partial gene recorded by (Laurimae *et al*, 2016). However, the sequence result for *sh4-1* gene in the comparison with database in GenBank showed that there was (99%) correspond with one isolate recorded in accession number (HM563031.1) in southern Iran (Harandi *et al*, 2016). The sequence result for *COI* gene in comparison with database in GenBank showed that there was (99%) correspond with isolate recorded in accession number (MF281540.1) (Yan *et al*, 2018), whereas in *COI* gene, the sequence results showed that there was (99%) identification with Estonia isolate (Kinkar *et al*, 2018). However, the sequence result for gene *CO1* in human showed that there was a (100%) isolated recorded in accession number (MH010310.1) (Shafiei *et al*, 2018), which the sequence result in comparison with database in GenBank revealed that there was (99%) identification with isolate recorded in accession number (FJ608748.1) (Calderini *et al*, 2018) in Italy. However, the other isolate from sheep showed that there was (100%) identification with Turkey isolate recorded in accession number (MF544127.1) (Oguz *et al*, 2018).

The sequence results for *ND1* gene in sheep samples showed that there was (100%) identification with isolate registered in accession number (MG672293.1) (Kinkar *et al*, 2018). The sequence results for *COI, CO1* for Donkey samples, in comparison with database in GenBank, showed that there was (100%) with Estonia isolate registered in accession number (KY766905.1) (Kinkar *et al*, 2017).

The phylogenetic analysis of *G6-7* gene in this study showed identification 97% with Iraqi isolate and 100% with many countries like Iran, turkey, Algeria, India and *sh4-1* gene in this study showed identification (99%) with Iran isolate (Harandi *et al*, 2016) and 100% with Iraqi isolate, 100% identification with many countries like Tunisia, Brazil, Turkey, India and Australia.

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