



Original article

Genotyping of *Taenia hydatigena* isolated from sheep and goats in KSA based on Cox1 geneMajed H. Wakid^{a,b,*}, Muslimah N. Alsulami^c^a King Abdulaziz University, Faculty of Applied Medical Sciences, Department of Medical Laboratory Technology, Jeddah, Saudi Arabia^b Special Infectious Agents Unit, King Fahd Medical Research Center, Saudi Arabia^c University of Jeddah, Department of Biology, College of Science, Jeddah, Saudi Arabia

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ABSTRACT

Sheep and goats are among other herbivorous animals that serve as intermediate hosts (containing the larval stage *Cysticercus tenuicollis*) of *Taenia hydatigena* tapeworm. This infection can lead to serious complications or cause death. The genetic diversity and epidemiological significance of cysticercosis due to *T. hydatigena* is poorly understood. We examined 11,651 goats and 23,542 sheep slaughtered at the municipal abattoir in Makkah, for *C. tenuicollis* infection. The resulted DNA sequences were compared with previously available sequences from different hosts. Phylogenetic analysis and Pairwise nucleotide variations of *cox1* gene were performed. Sheep and goats revealed infection rates of (4.95%) and (4.75%) respectively. DNA sequence analysis of all isolates from both sheep and goats showed that the total haplotypes number was 7. *T. hydatigena* population with high haplotypes diversity values. The nucleotide diversity was low, while Tajima's D and Fu's tests were negative (with no statistical significance). The present work will give valuable information regarding the prevalence and implementation of control and prevention measures of *C. tenuicollis*.

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1. Introduction

The adult worms of *Taenia hydatigena* inhabit the small intestine of dogs and other canids final hosts such as wolves and foxes (Jenkins et al., 2014). A wide range of herbivorous animals (especially ruminants) including cattle, sheep, goats, camels, and pigs are suitable intermediate hosts.

The seriously affected sites with *Cysticercus tenuicollis* larval stage are the liver, the omenta and the mesenteries in the intermediate hosts (Samuel and Zewde, 2010; Saulawa et al., 2011; Oryan et al., 2012; Mekuria et al., 2013). Hepatitis cysticercosa, which contributes to hemorrhagic and fibrotic tracts and serofibrinous peritonitis, can be caused by the movement of these cysticerci into the liver. In several severe cysticercosis cases, the larvae were able to cause eosinophilic liver infiltration and fatal intense inflamma-

tion (Blazek et al., 1985; Gholami, 2018; Darzi et al., 2002; Fentahun et al., 2012). On the other hand, liver damages associated bacterial growth were reported (Popova and Kanchev, 2013).

Moderate to serious infections may present with diarrhea, anemia, and a decline in growth rate, leading to higher costs of feeding. Animals infected may also become frail and highly susceptible for other infections (Blazek et al., 1985).

C. tenuicollis infections have been paid greater attention due to its economic importance as a result of the condemnation of offals containing these larvae, particularly in resource-poor countries (Wondimu et al., 2011). *T. hydatigena* is prevalent worldwide, with a global distribution ranging from 0.1 to 32.0% (Scala et al., 2015; Braae et al., 2015; Nguyen et al., 2016).

Most of the cases in Africa and Europe have been identified among sheep and goats, whereas in Asian and American countries the prevalence was higher in pigs (Wondimu et al., 2011; Payan-Carreira et al., 2008).

The livestock sector in Saudi Arabia is a significant one. In 2018, the estimated head number of livestock in Saudi Arabia was about 135 million (The General Authority for Statistics).

To date, there are few studies concerned with *C. tenuicollis* in Saudi Arabia. In 1997, a study was conducted in Qassim region examined 6358 animals slaughtered at an abattoir in Buraydah.

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The overall rate of parasite infection was 11.3%. The prevalence of *T. hydatigena* was 1.25% (El-Metenawy, 1999). Another study in Jeddah has shown that prevalence in goats and sheep are 23.4% and 0.45% respectively (Bakhrabih, 2016).

Several molecular approaches have been established to differentiate *Taenia* species (Jia et al., 2010). Mitochondrial DNA sequence analyses have been commonly used as genetic markers to analyze the molecular characterization of taeniid cestodes, as they experience low recombination rates. There is little data about the genetic diversity of *T. hydatigena*. Even though the globally carried-out studies mainly investigated partial sequences of *cox1* (cytochrome *c* oxidase subunit 1) and *nad1* (NADH dehydrogenase subunit 1). Mitochondrial genes in variety of hosts have recorded substantial levels of genetic diversity among *T. hydatigena* populations (Kedra et al., 2001; Rostami et al., 2015; Boufana et al., 2015; Omar et al., 2016).

No previous studies investigated *C. tenuicollis* infection rates and molecular characterization in Saudi Arabia. So, the main aims of the current study were to assess the distribution of *C. tenuicollis* among sheep and goats in Makkah, Saudi Arabia and to identify the molecular characteristics of mitochondrial DNA coding for *cox1* gene. Data from the present work will give valuable information regarding the prevalence of *C. tenuicollis* in Saudi Arabia. Moreover, molecular characterization of these cysts is vital for implementation of control and prevention measures.

2. Materials and methods

The study was performed on slaughtered animals and approved by the Ethics & Research Committee of the Faculty of Applied Medical Sciences, King Abdulaziz University (FAMS-EC2019-13).

2.1. Sample collection

The present study examined 11,651 goats and 23,542 sheep slaughtered at the municipal abattoir of Makkah, Saudi Arabia during the period from January to April 2020. These slaughtered animals were males and females, among two age groups: young (1 year old or less) and adult (older than 1 year).

2.2. Parasitological analysis

The visceral organs of the slaughtered animals were examined for the presence of *C. tenuicollis* cysts, relied on the characteristic features of the long-necked single scolex, translucent cyst fluid and armed hooked rostellum. The cysts were collected and washed with saline and transferred into sterile containers at -20°C for further examinations.

2.3. DNA extraction, PCR and DNA sequencing

DNA was extracted from the cysts using DNA tissue extraction kit from Jena Bioscience (CAT NUM LOT 116.517) following the manufacturer's instructions. A primer pair targeting *cox1* gene, forward (5'-CATTITTTGATCCAT TAGGTGGTGG) and reverse (R5'-ACATAAGTACAAT CATCTACTCAC) were used.

PCR reaction (50 μl) was performed using thermal cycler (Mastercycler[®] Personal, Eppendorf). The PCR settings were: initial denaturation for 5 min at 95°C , 36 cycles of 40 sec at 95°C , 45 s at 46°C , 1 min at 72°C , and final extension for 10 min at 72°C . Amplified PCR products were subsequently sequenced using ABI Hitachi Applied Biosystems 3130 DNA Genetic Analyzer DNA Sequencer. The resulted DNA sequences were compared with DNA sequences of *C. tenuicollis* of different hosts and countries that are deposited previously in the GenBank using BLAST system. Mul-

iple alignments were done using MUSCLE (Multiple Sequence Comparison by Log-Expectation) alignment of Mega X software. Phylogenetic analysis and Pairwise nucleotide variations of *cox1* gene were performed using the neighbor joining (NJ) method of the Mega X program. DnaSP 5.10 software was used to estimate the haplotype numbers (H), haplotype diversity (Hd), nucleotide diversity (π), Tajima's D, and Fu & Li's statistics value (Librado and Rozas, 2009). Tajima's neutrality test was considered significant when $P > 0.10$. To analyze the Median Joining network of haplotypes, we used network 4.6 program.

2.4. Statistical analysis

All frequency distributions data and demographic results were analyzed using Statistical Package for Social Sciences, SPSS, V. 22. P -value < 0.05 was assumed to be statistically significant.

3. Results

Examination of the visceral organs revealed that 4.95% (1167/23542) of sheep and 4.75% (554/11651) of goats, were infected with *C. tenuicollis* cysts. Infection was more prevalent in males of both populations (sheep and goats) and younger aged animals showed higher rates of infections. There was no significant association between age, sex, and the prevalence of cysticercosis (Table 1).

PCR amplification was successfully obtained for all examined cysts (30 goats and 30 sheep) using primers targeting *cox1* gene. The amplified DNA fragments size was 943 bp. A representative gel for the PCR results is presented in Fig. 1.

Sixty PCR amplified DNA fragments of the *C. tenuicollis* cysts were used for DNA sequence analysis. Overall, 10 different isolates were successfully amplified for *cox1* gene. BLAST 2 software was used for alignment of our complete DNA sequencing of the *cox1* gene with other GenBank-accessible gene sequences. The nucleotide sequences similarity was $>99\%$.

Phylogenetic relationship of all *C. tenuicollis* isolates from sheep and goats calculated by NJ method from the partial *cox1* gene nucleotide sequences revealed a total of 10 isolates. The variance of isolates ranged from 0.0006 to 0.681, which means low nucleotide diversity. One isolate was unique for goats only and two isolates were unique to sheep, while other isolates were common for both sheep and goats (see Table 2).

As regards the analysis of complete *cox1* gene of goats, we recognized 15 polymorphic sites, 10 of them were parsimony informative. The number of haplotypes (H) was 7 with haplotype diversity of 0.852 and nucleotide diversity 0.00235. Sheep isolates had 5 polymorphic sites, of which two were parsimony informative. The number of haplotypes (H) observed was 6 with 0.6878 haplotype diversity and 0.00225 nucleotide diversity.

The overall number of haplotypes in both sheep and goats was 7. The frequency of the major haplotype was (29/56%) in both populations (Fig. 2). There was one mutational step between the major haplotype and each of the other six haplotypes. There were 6-point mutations between the major core haplotype and the other haplotypes, including 3 singleton variable sites at positions 9675 9730 9950 and 3 parsimony informative sites (PIS) at positions 9727 9793 9799.

The overall haplotype diversity was high with low nucleotide diversity (Table 3). This means that although we have several haplotypes, but they are very similar with few nucleotide differences. *T. hydatigena* population from both goats and sheep showed negative Tajima's D and Fu's F_s (with no statistical significance), which may be explained by new population expansion and that DNA sequence is evolving neutrally not under directional selection.

Table 1
Prevalence of *C. tenuicollis* among examined animals.

		Number examined		No. (%) infected		P value
		Sheep	Goats	Sheep	Goats	
Sex	Male	17,531	8419	936 (3.98%)	312 (2.68%)	0.09
	Female	6011	3232	231 (0.98%)	242 (2.07%)	
Age	Young	19,541	9221	889 (3.78%)	413 (3.54%)	0.07
	Adult	4001	2430	278 (1.18%)	141 (1.21%)	
	Total	23,542	11,651	1167 (4.96%)	554 (4.75%)	

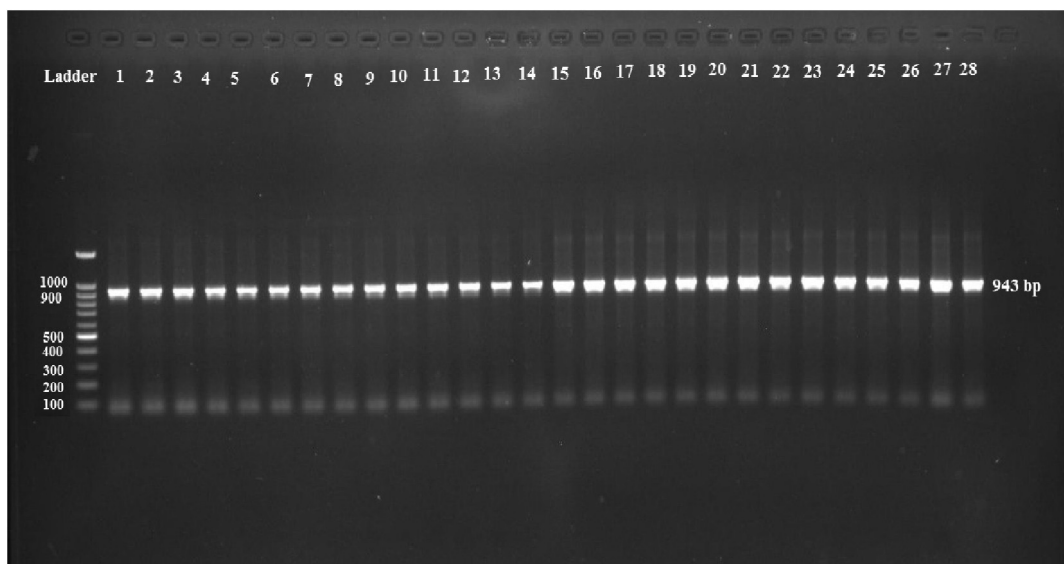


Fig. 1. Agarose gel electrophoretic analysis of *C. tenuicollis* samples amplified by conventional PCR showing the 943 bp. DNA ladder 100 bp.

Table 2
Pairwise nucleotide differences between the haplotypes of the (*cox1*) gene DNA sequences. Analysis was conducted using Maximum Composite Likelihood method.

	H	H	H	H	H	H	H
H1							
H2	0.00423						
H3	0.00541	0.00541					
H4	0.00370	0.00169	0.00270				
H5	0.00494	0.00254	0.00541	0.00370			
H6	0.00494	0.00507	0.00541	0.00370	0.00494		
H7	0.00556	0.00592	0.00541	0.00432	0.00556	0.00556	

Phylogenetic tree to compare the DNA sequences of *C. tenuicollis* cysts obtained in the present study with other GenBank-accessible sequences was constructed using NJ method, (Fig. 3). DNA sequences of *C. tenuicollis* isolates from china, Nigeria and Sudan were used. *Echinococcus granulosus* was used as outgroup. Sequences from the GenBank database indicating that all isolates were clearly *T. hydatigena* and presented significant distances from related *Taenia* species (*T. saginata*, *T. multiceps*, and *T. asiatica*). All genetic sequences are available at [<https://www.ncbi.nlm.nih.gov/genbank/>]. GenBank accession numbers are: MZ314830, MZ318039, MZ318040, MZ318041, MZ318042, MZ318043, MZ318044, MZ318045, MZ318046, MZ318047.

4. Discussion

Cysticercosis caused by *C. tenuicollis* (the larval stage of the canine tapeworm *T. hydatigena*) is a worldwide parasitic disease affecting herbivorous animals due to invasion of tissues. The infection is manifested by hepatitis cysticercosa in infected intermedi-

ate herbivorous hosts and could be fatal in severe cases. Global concern has been paid to *C. tenuicollis* because of its financial impact due to condemnation of offal's containing these larvae, especially in low economic countries (Oryan et al., 2012; Getaw et al., 2010).

Identification of *C. tenuicollis* in slaughterhouses is based mainly on inspection of meat and organs. No reports are available about the prevalence and molecular characterization of the *C. tenuicollis* in slaughtered animals in Saudi Arabia.

A total of 23,542 sheep and 11,651 goats slaughtered at the municipal abattoir of Makkah, Saudi Arabia were examined for *C. tenuicollis* infection. The overall infection rates in sheep and goats were 4.96% and 4.75 respectively. In Saudi Arabia, only one survey has been conducted in Qassim region in 1997 by EL-Metenawy shown that cysticercosis caused by *T. hydatigena* was 1.7% (El-Metenawy, 1999). The results of the present study is lower than prevalence reported from other countries (Saulawa et al., 2011; Mekuria et al., 2013; Azmy, 2014; Radfar et al., 2005). This low prevalence may be due to the grazing roles and management sys-

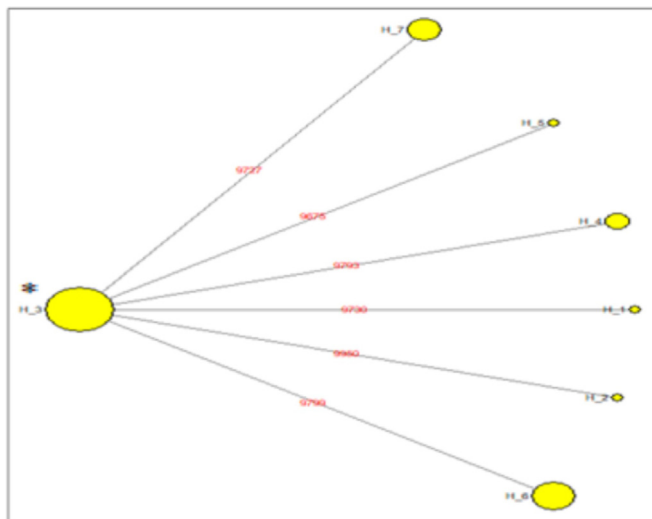


Fig. 2. Median-joining network, showing 7 haplotypes based on *cox1* gene. (*) represents the founder haplotype. The size of circle corresponds frequency of haplotype. There is only one mutation step between the founder haplotype and the rest of haplotypes.

Table 3
Results from DNA sequence analysis of sheep and goats isolates using DnaSP program.

	<i>Cox1</i> gene (1620)		
	Sheep	Goats	Overall
Number of mutations	5	15	6
Parsimony informative sites	2	10	3
Haplotypes number	6	7	7
Haplotype diversity	0.6878	0.852	0.6714
Nucleotide diversity	0.00232	0.00235	0.00226
Tajima's test	-0.924695	-0.952136	-0.899074
Fu's test	-2.217	0.476	-2.482

tem in our country. In addition, slaughter procedures and proper disposal of infected organs are carried out in the slaughterhouses. This can effectively interrupt the parasite life cycle continuity between the definitive and intermediate hosts. Moreover, the weather of Saudi Arabia, which is characterized by long, hot, dry summer, may largely contribute to the low prevalence of *C. tenuicollis*, due to the significant effect on the viability of parasite eggs (Torgerson and Heath, 2003).

There was no significant difference in the infection rates among different sex and age groups of animals. This could be attributed for the exposure to similar infective eggs of *T. hydatigena*.

Although *C. tenuicollis* cysticercosis causes severe damage in livestock production in endemic localities (Saulawa et al., 2011; Christodoulou et al., 2008; Scala et al., 2016), the genetic diversity and its epidemiological significance is poorly understood (Braae et al., 2015; Omar et al., 2016).

Mitochondrial DNA is characterized by no recombination, maternal inheritance pattern, conserved regions, rapid mutation rate and a relatively high rate of evolution. Therefore, it has been widely used in investigation of the genetic variation of metazoans (Brown et al., 1979; Mueller et al., 2004; Shen et al., 2010; Wei et al., 2010). Our analysis of complete sequences for *cox1* gene revealed a considerable degree of genetic variation of *T. hydatigena* isolates from sheep and goats collected from a slaughterhouse in Makkah, Saudi Arabia, in contrast to the commonly employed partial gene sequences (Braae et al., 2015; Boufana et al., 2015; Adwan et al., 2018; Kilinc et al., 2019).



Fig. 3. Phylogenetic relationship calculated by NJ method from the partial *cox1* gene nucleotide sequences. *E. granulosus* represents the out-group strain. The scale bar corresponds nucleotide substitutions per site.

We analyzed a 1620 bp of the gene for a total of 60 isolate of sheep and goats. Over the whole data set, we identified 3 parsimony informative sites and 3 mutations (not parsimony informative) resulting in the detection of 7 haplotypes. One of these haplotypes was found to be common in both sheep and goats and appeared frequently (29/60) on the overall data set. In addition, another haplotype was identified only in goats, while 2 haplotypes were unique to sheep. The shared haplotypes represented 95% of the total number of isolates. The number of haplotypes was comparable between sheep and goats. 6 haplotypes were isolated from sheep and 7 form goats. We noticed elevated haplotype diversity in both populations, ranging from 0.67 to 0.86, which is high in comparison to many other species. In accordance with the present study, high haplotype diversity values have been reported in other haplotypes of *T. hydatigena* including the Nigerian, Italian, Iranian and Sudanese haplotypes (Boufana et al., 2015; Ohiolei et al., 2019). In contrast, it is higher than the haplotype diversity reported in Palestine *T. hydatigena* populations of sheep (Boufana et al., 2015; Adwan et al., 2018). Lower prevalence

and infection rate of *C. tenuicollis* in smaller areas with low small ruminant population may influence the genetic diversity of *T. hydatigena* (Boufana et al., 2015; Adwan et al., 2018). Higher genetic diversity are common in higher prevalence and infection rate of cysticercosis with higher population of small ruminants (Boufana et al., 2015).

The nucleotide diversity was relatively low; $\pi = 0.00232$ for sheep and $\pi = 0.00235$ for goats. This finding in addition to the high haplotype diversity can be a sign of a rapid expansion of a small effective population size (Avise, 2001). Several statistical tests depend on the distribution of pairwise differences between sequences within populations are used to test for the selective neutrality of mutations and to detect population expansion (Ramos-Onsins and Rozas, 2002). We applied Tajima's D test (Tajima, 1989) for the allele frequency distribution of discrete nucleotide sites. Fu's FS test (Fu, 1997), is based on the distribution of alleles or haplotypes. In our study, the Tajima D test and the Fu FS test revealed negative values for all populations, which are indicative of recent population expansion.

5. Conclusions

To the best of our knowledge, this is first study in Saudi Arabia concerns the genetic diversity of *T. hydatigena* using complete mitochondrial gene sequences. Our study proposes the possible occurrence of host-specific strains. The lack of entire *T. hydatigena* mitochondrial *cox1* sequences in the GenBank restricted us from extensive comparison and interpretation. For effectively future applications, greater understanding of genetic diversity and the potential epidemiological significance, we recommend expansion and public availability of mitochondrial genome data for *T. hydatigena*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

M. W. and M.A. designed the study, supervised the collection of samples and the practical work, analyzed the data of the results, prepared and revised the manuscript.

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