Genotypes of Echinococcus Species from Stray Dog in Wasit /Iraq

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Abstract
The dog tapeworm Echinococcus granulosus is a cosmopolitan parasite. The adult worms reside in the small intestine of their definitive hosts (dogs). In this study presents the results of a study of feces of 110 dogs from AL Kut center, AL - Numaniya, AL Hayy, Jassan, AL- Zubaydiya & AL-dubuni regions for Echinococcus granulosus, collected in from January 2022 to May 2022. Eggs of Taeniia spp. were found using copro-pcr methods. Based on the analysis of the nucleotide sequences of 40 egg samples, 22 samples were identified as E. granulosus. Based on the (cox1) gene, the G1, G2, G3, G5and G6 genotype was established, genotypes G1 and G3 of E. granulosus show that sheep strain and buffalo strain are unambiguously circulating in the region. These samples were deposited in the NCBI GenBank database. This study is considered the first to identify the genotype of Echinococcus spp. in Wasit/ Iraq.
Keywords: Echinococcosis, dogs, coproPCR, genotypes

1. Introduction

Dogs are the first species to have been domesticated and share a close cultural, social and economic For association with humans [1]. Domestication of dogs has been shown to provide many benefits to humans [2]. example, pet ownership is associated with a decreased prevalence of depressive symptoms [3]. It has been reported that pet owners visit their doctor less often, use fewer medications and have lower blood pressure and cholesterol levels than non-pet owners [4]. Dogs have roles in the management of many psychological, psychiatric and biomedical conditions in humans [5,6]. However, dog domestication in particular, the overpopulation of stray dogs can have negative impacts on public health and animal welfare.

A stray dog is defined as any dog in a public area that is not under direct human control. Therefore, this term encompasses unowned and community-owned dogs but excludes dogs on leashes or under direct human control at the time of a survey (WSPA, 2009). Stray dogs can be either previously owned dogs [7,8] or can be feral dogs (wild dogs) that have never been owned [9]. Factors such as easy availability of food, lack of predators, low number of competitors, and the ease with which a breeding partner can be found can lead to rapid increases in stray dog populations [10].

In contrast to the health benefits provided by pet dogs, stray dogs contribute to environmental pollution, dog bite incidence and can act as reservoirs of many important zoonotic parasites (for example, Toxocara, Ancylostoma and Echinococcus) via faecal contamination of soil and water [11–13], and infectious diseases (for example, rabies, salmonellosis). The faecal shedding of pathogens by stray dogs contaminates the environment [14], which is of substantial public and animal health concern. Stray dogs also contribute to incidents such as bites and accidents and damage wildlife populations [15–18]. In addition, stray dogs have a substantial negative economic impact at tourist destinations [19]. High numbers of stray dogs are due to large amounts of edible waste available on the streets, cultural tolerance of stray dogs and a lack of consistently employed sustained birth control programs [20]. Genetic diversity in E. granulosus species has been reported since long and it is suspected that this interspecies variation may influence the infectivity and various other characteristics i.e. morphology, specificity of host and epidemiology [21]. Mitochondrial DNA sequence analysis has identified ten heterogeneous groups (G1-G10) of variants in E. granulosus. Among them, three genotypes, the G1-G3 (G1-G2 found in sheep while G-3 in buffalo), are grouped in the species E. granulosus sensu stricto while other genotypes are E. equinus which is found in horses (G4), E. ortleppi in cattle (G5), E. Canadensis (G6–10 found in camels, pigs and cervids, respectively) and the lion strain (E. felidis) [22;23;24] .

2. Study regions of dog

Located at an elevation of 16.75 meters (54.95 feet) above sea level, Wasit has a Subtropical desert climate. The city's yearly temperature is 29.2°C sometimes up to 47 degrees, Humidity 24.37%. Wasit typically receives about 20.6 millimeters (0.81 inches) of precipitation and has 34.09 rainy days (9.34% of the time) annually. The cold months from November to March. The study area to dog be an AL Kut center, AL - Numaniya , AL Hayy, Jassan, AL-Zubaydiya & AL- dubuni . The regions evaluated in this study are show in Figure 1.

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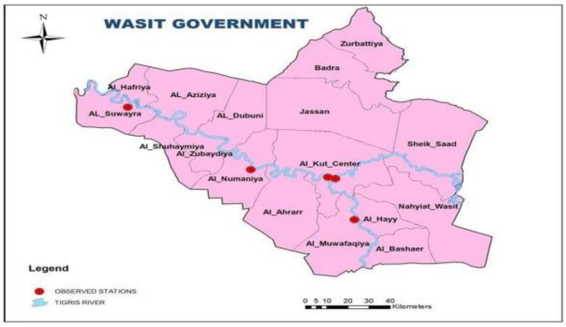

Figure: (1) :Map of Wasit Government. (Directorate of the Environment for Wasit province).

3. Materials and Methods

Faecal sample collection. This research was carried out between the months of January and May of 2022. On the feces of 110 stray dogs collected from various parts of Wasit Province. The samples were collected at random in public places. On the same day, the feces were brought to the laboratory and kept at −80°C to keep the eggs inactive until they were used (25). The feces were macroscopic examined by observing the visible outer surface under sufficient light and inspecting the inner surface by crumbling the feces with a glass rod to determine the presence of cestodes. The method of formalin-ether concentration was used (26). Using a microscope, the presence of taeniid (Taenia spp., Echinococcus spp.) eggs was determined. All of these samples, including taeniid eggs, were chosen for further molecular research.

Copromicroscopic and Molecular Analyses

The formalin-ether sedimentation method has been modified [27,28]. Was used to detect taeniid eggs in fecal samples. For proper emulsification, approximately 1 g of stool was collected with a spatula and emulsified in 3 mL of 10% formalin in a mortar using a pestle. The stool emulsion was poured into a 15 mL centrifuge tube through a fine mesh of 250 m. 2 mL of 10% formalin was used to wash the stool through the gauze, 3 mL of ethyl acetate, in place of the original method's diethyl ether [29], was added to the centrifuge tube's contents and mixed, For 5 minutes, the mixture was centrifuged at 448 g. Following centrifugation, four layers were formed in the tube: an ethyl acetate layer, a debris plug, a formalin layer, and the sediment. After removing the top three layers, the sediment was thoroughly mixed, on a glass slide, two droplets of sediment were added together with one drop of iodine solution, and a cover slip was then put on top, The coverslip was then thoroughly looked at using an optical microscope (4x, 10x and 40x). Molecular tests were used to further identify the discovered taeniid eggs, Molecular analyses were used to identify the taeniid eggs that were discovered. Presto™ Stool DNA Extraction Kit was used to extract genomic DNA, and a multiplex polymerase chain reaction protocol was followed as already described [30].The total reaction mix volume was 25 μL: 12.5 μL Master Mix; 2 uL of Primer Cest (20 pmol/μL) and of Primer (20pmol/μL) ; 7.5μL nuclease-free water and 3 μL of template DNA (5 ng DNA per tube). Primers amplify fragments of the mitochondrial cytochrome oxidase subunit 1 (cox1) gene specific for E. granulosus s.l. (PCR Cox1 gene F (GTTTAGGGGCTGGTGTTGGT) R (TGAGCCACCACAAACCAAGT) 772bp, Nested PCR Cox1 gene F (TCTCTGCATTTGGCTGGTGT) R CCGTAACTCCCCCAAACGTA) 619bp) . Agarose gel electrophoresis was performed on precast gels (E-Gel™ EX Agarose Gels, 1.5% Agarose, (iNtRON, Korea). using the E-Gel™ Power Snap Electrophoresis System . DNA ladder (iNtRON, Korea)) was loaded onto the agarose gel for size determination of the PCR products.

4. Statistical Analysis

The Statistical Package for Social Sciences (SPSS) version 24 was used to examine the data and Microsoft Office Excel 2019, by using the Chi-squire, Level of significant was consider at P-value ≤ 0.05.

5. Results and Discussion

Coprology studies using microscopes of faecal samples from a dog were used to determine the presence of Taeniid eggs by indirect concentration techniques i.e., sedimentation methods (Figure 2).

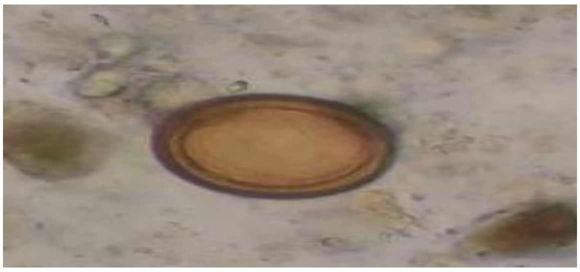

Figure 2. Taeniid eggs (Echinococcus granulosus) detected in dog faecal samples (sedimentation technique) 40×.

Out of 110 fecal samples over a five-month period from January 2022 to June 2022. Collected and observed by conventional methods (floatation method and formalin-ether concentration technique) only 36% (40/110) samples were found positive for Taenia -type eggs under microscope on the basis of morphology of eggs. Figure (3-1).

PCR product and gel electrophoresis

Extraction of the DNA parasites from dog feces , which was directed on 40 samples was affirmed typical values of the concentration DNA the extracted genomic DNA from feces samples was checked by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).

total 40 of different E. granulosus DNA isolates were studied by using PCR amplification of partial mitochondrial cytochrome C oxidase subunit1 (Cox1) with PCR product of 619 bp, that by using the multiple Nested PCR Cox1 gene, Among the results were positive for 22(55%) them, 18 negative samples appeared, these negative samples were excluded from the following tests, Electrophoresis was used to visualize them using a 1.5% gel concentration for the best separation of large molecular weight DNA ,The PCR products of the isolates by using gel electrophoresis revealed major banding patterns ,figure (3) showed a good qualification of extracted genomic DNA.

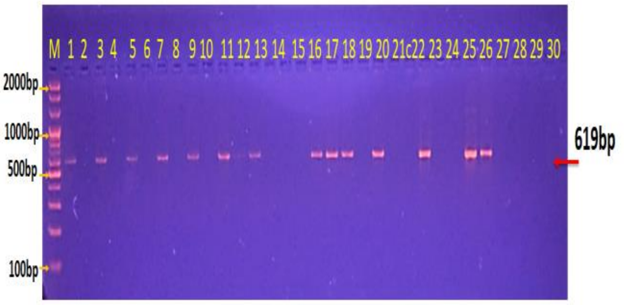

Figure (3): Agarose gel electrophoresis image that showed the Nested PCR product analysis of Echinococcus granulosus mitochondrial cytochrome oxidase subunit 1 (COX1) gene from extracted DNA of dog's feces samples. Where M: marker (2000-100bp). The lane (1-30) showed some positive Echinococcus granulosus mitochondrial (COX1) gene from dog samples at (619bp) PCR product.

Copro-polymerase chain reaction has higher sensitivity compared to centrifugal fecal flotation in the diagnosis of taeniid cestodes, especially Echinococcus spp ,CoproPCR has promising prospects for use in Veterinary clinics and diagnostic laboratories to detect taeniid cestode infections because of its higher sensitivity than faecal flotation methods [31] . This agreement with Oguz et al.,[32] in Turkey, Lu et al., [33] in China , Mirbadie et al .,[34] The better effect of the COX1 gene in was the identification of Taenia in species and interspecies.

The Mt-cox1 and EgG1 Hae III genes have recently been used in the development of a copro-PCR for the identification of E. granulosus, No cross-amplification with Taenia hydatigena, Dipylidium caninum, Taenia ovisor, and E. multilocularis has been reported [35].

The study involved the selection of 20 samples from a total of 22 nested pcr positive samples, where the most obvious samples were chosen during gel electrophoresis operations. The samples were then analyzed to determine the genetic sequence of DNA, and the samples were sent to Macrogen Company/Korea to determine the DNA sequence. The results were received by email then analyzed using genious software after being sent to Macrogen Company in Korea that Sequencing Technology, automated DNA sequences. Utilizing Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0), multiple sequence alignment analysis based on ClustalW alignment analysis, and the phylogenetic tree UPGMA method, the DNA sequencing analysis (phylogenetic tree analysis) was carried out. The evolutionary distances were calculated using the Maximum Composite Likelihood method. Finally identified Echinococcus granulosus genotypes isolates were submitted into of NCBI-Genbank to get Genbank accession number table (3 - 4).

The results of the analysis of the sequence of the nitrogen bases of the polymerase chain reaction and the E.granulosus parasites showed that most of the samples studied were consistent with those recorded in the National Center for Biotechnology Information (NCBI) with a ratio of 99-100%, by used Basic Local Sequence analysis tool appear in table. (1).

Table (1): NCBI -BLAST Homology sequence identity between local Echinococcus granulosus Dogs isolates with NCBI-BLAST Echinococcus granulosus related Genotypes isolates.

Local isolate	Genbank accession number	NCBI-BLAST Homology Sequence identity			
		NCBI BLAST identity Genotype	Country	Accession number	Identity (100%)
Dog No.1	OP106966	Genotype 1	Turkey	MN732819.1	99%
Dog No.2	OP106967	Genotype 3	Turkey	MW421883.1	100%
Dog No.3	OP106968	Genotype 1	Turkey	MN732819.1	99%
Dog No.4	OP106969	Genotype 3	Turkey	MW421883.1	99%
Dog No.5	OP106970	Genotype 6	Iran	LC476634.1	100%
Dog No.6	OP106971	Genotype 1	Turkey	MN732819.1	99%
Dog No.7	OP106972	Genotype 3	Turkey	MW421883.1	100%
Dog No.8	OP106973	Genotype 1	Turkey	MN732819.1	99%
Dog No.9	OP106974	Genotype 3	Turkey	MW421883.1	99%
Dog No.10	OP106975	Genotype 1	Turkey	MN732819.1	100%
Dog No.11	OP106976	Genotype 3	Turkey	MW421883.1	99%
Dog No.12	OP106977	Genotype 1	Turkey	MN732819.1	100%
Dog No.13	OP106978	Genotype 2	Palestine	KC109660.1	99%
Dog No.14	OP106979	Genotype 1	Turkey	MN732819.1	99%
Dog No.15	OP106980	Genotype 6	Iran	LC476634.1	100%
Dog No.16	OP106981	Genotype 5	India	JX854035.1	99%
Dog No.17	OP106982	Genotype 1	Turkey	MN732819.1	100%
Dog No.18	OP106983	Genotype 5	India	JX854035.1	99%
Dog No.19	OP106984	Genotype 1	Turkey	MN732819.1	99%
Dog No.20	OP106985	Genotype 5	India	JX854035.1	100%

Phylogenetic tree of the strains

The genetic tree analysis of the isolates of the studied samples based on the partial sequence of the mitochondrial Cox1 gene of local isolates of Echinococcus granulosus in definitive hosts showed the genetic relationship between current strains and the strains in other countries, especially neighboring

countries.
Evolution distances, which were measured using the similarity method for the maximum compound, were calculated by the UPGMA tree tool (MEGA version 6.0).
The local isolates of E. granulosus showed affinity in varying proportions with the global isolates registered in the BLAST-NCBI gene bank, where the numbered local isolates (OP106966, OP106968, OP106971, OP106973, OP106975, OP106977, OP106979, OP106982, and OP106984) (Dog No.1 , No.3, No.6 , No.8 , No.10, No.12, No.14, No.17and No.19) showed the closest similarity to E. granulosus isolates belonging to strain G1 with accession number (MN732819.1) with homology sequence identity was (99%, 99%, 99%.99%, 100%, 100%, 99%, 100%, 99%) respectively. Phylogenetic analysis revealed a robust tree associating our isolate of G1 genotype with the same sister group as a variety of G1 genotype (common sheep strain) sequences from different geographical regions of the world, although it was more genetically related to the Turkey isolate.
The numbered isolate (OP106978) (dog N.13) showed the closest similarity to E. granulosus isolate belonging to strain G2 numbered with accession number (KC109660.1) with homology sequence identity was(99%) Phylogenetic analysis revealed a robust tree associating our isolate of G2 genotype with the same sister group as a variety of G2 genotype (common buffalo strain) sequences from different geographical regions of the world, although it was more genetically related to the Palestine isolate.
The numbered isolate (OP106967, OP106969, OP106972, OP106974, OP106976) (Dog No.2, No.4, No.7, No.9and No.11) showed the closest similarity to E. granulosus isolate belonging to strain G3 numbered with accession number (MW421883.1) with homology sequence identity was (100%,99%,100%,99% and 99%) respectively, Phylogenetic analysis revealed a robust tree associating our isolate of G3 genotype with the same sister group as a variety of G3 genotype (common buffalo strain) sequences from different geographical regions of the world, although it was more genetically related to the Turkey isolate .
The numbered isolates (OP106981, OP106983, OP106985) (Dog No.16, No.18 and No.20) showed the closest similarity to isolates E. granulosus belonging to strain G5 numbered with accession number (JX854035.1) with homology sequence identity was (99%,99% and 100%) respectively Phylogenetic analysis revealed a robust tree associating our isolate of G5 genotype with the same sister group as a variety of G5 genotype (common cattle strain) sequences from different geographical regions of the world, although it was more genetically related to the India isolate
The numbered isolates (OP106970, OP106980) (Dog No.5 and No.15) showed the closest similarity to isolates E. granulosus belonging to strain G6 numbered with accession number (MN732819.1 and LC476634.1)) with homology sequence identity was(100%) respectively Phylogenetic analysis revealed a robust tree associating our isolate of G6 genotype with the same sister group as a variety of G6 genotype (common pig strain) sequences from different geographical regions of the world, although it was more genetically related to the Turkey and Iran isolate , at total genetic heterogeneity (0.16-0.0) as Shown in the figure (4).

Genotyping Distribution of *Echinococcus granulosus* in Wasit province

The results were the sheep strains (G1) nine Distributed in to AL- Numaniya ,AL Kut center, Jassan, AL- Zubaydiya), one G2 sheep strain (in AL Kut center), five buffalo strain (G3) (in AL- Numaniya , Jassan , AL Hayy), three G5 cattle strain (in AL-Zubaydiya and AL- dubuni , Jassan, AL Kut center) , two camel strain (G6) (in AL Kut center, AL-Zubaydiya) .table (2).
Molecular studies revealed that E. granulosus s.s. clade (G1, G3) was involved in most cases of infection, among which the G1 (dog–sheep cycle) genotype was the most diverse and prevalent widespread in most parts of Wasit, 9 of 20 (0%) cases in final hosts, and dominant in 4 region, particularly in AL Kut center, and G3(Buffalo strain),5 of 20 (0%) cases in final hosts, and dominant in 3 region, particularly in Jassan indicating that G1 and G3 is viable for transmission.

Table (3-6): Genotyping Distribution of Echinococcus granulosus in Wasit province.

Genotypes	Region	NO.	%	Total%
G1(sheep strain)	AL- Numaniya AL Kut center Jassan AL- Zubaydiya &AL- dubuni	2 4 1 2	10% 20% 5% 10%	45%
G2(sheep strain)	AL Kut center	1	5%	5%
G3(buffalo strain)	AL- Numaniya Jassan AL Hayy	1 3 1	5% 15% 5%	25%
G5(cattle strain)	AL- Zubaydiya Jassan AL Kut center	1 1 1	5% 5% 5%	15%
G6(camel strain)	AL Kut center AL- Zubaydiya	1 1	5% 5%	10%

In this study, existence of genotypes G1 and G3 of E. granulosus show that sheep strain and buffalo strain are unambiguously circulating in the region. In this study the G6 camel strain, G2 sheep strain and G5 cattle strain was first found in a stray dog. this seems to indicate that the role of secondary intermediate hosts (buffalo/ goat/sheep/cattle) which can potentially play a role in the maintenance of genotypes strain dog life cycle[36;37]. The G1 isolates could be alike in different regions since the intermediate hosts are transported from one farming region to another (slaughterhouse or livestock market) [38].

Dogs usually become infected with E. granulosus genotype G1 after eating the discarded offal of wild or domestic ungulates, since feeding the viscera of wild and domesticated animals to dogs is commonly practiced, dogs may carry the parasite into urban environments following hunting trips in rural areas. Free-roaming stray dogs that move between rural and urban areas can also be a source of contamination [39;40]. It is worth mentioning that the genotyping of adult Echinococcus strains can indicate the scale of parasite biology in the region, while this shows that the intermediate hosts may acquire the infection from neighboring countries/provinces due to their immigrations and importations whereas, the stray dogs are sympatrically limited to an indigenous life [41;42].

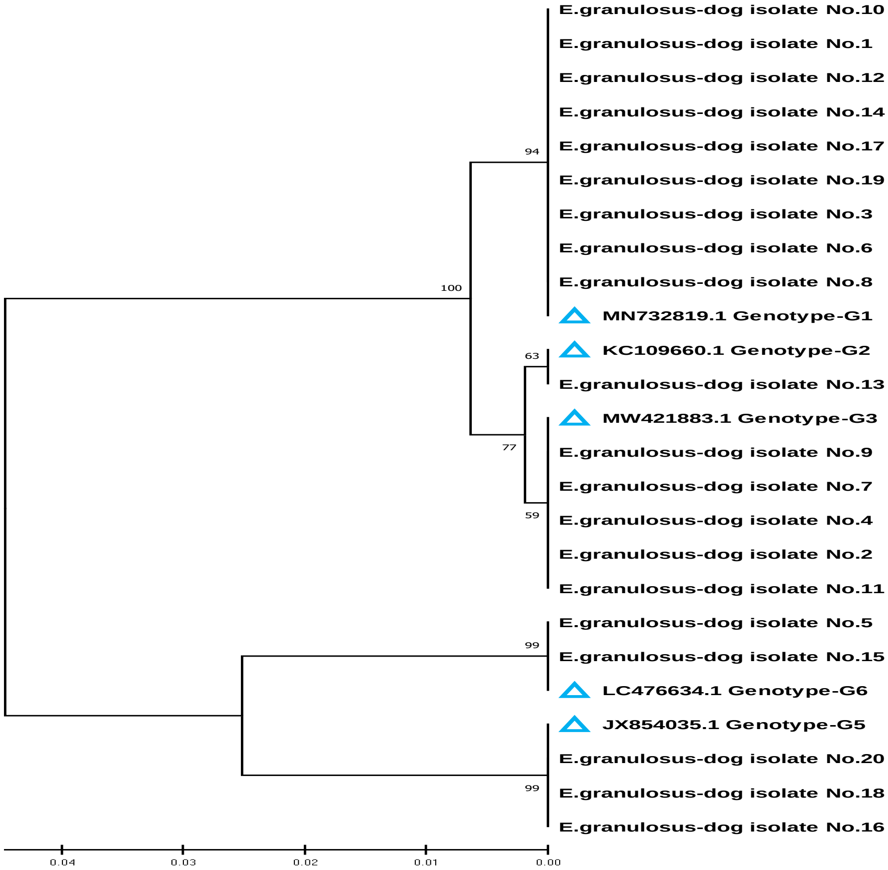

Figure (4): Phylogenetic tree analysis based on the Mitochondrial Cox1 gene partial sequence that used for Dogs Echinococcus granulosus typing detection. The phylogenetic tree was constructed using the evolutionary distances were computed using the Maximum Composite Likelihood method (UPGMA tree) in (MEGA 6.0 version). The local E. granulosus Dogs isolates (No.1-No.20) were show closed related to NCBI-Blast E. granulosus isolate (Genotype 1 , Genotype 2, Genotype 3, Genotype 5, and Genotype 6) at total genetic change (0.04-0.01).

6. Conclusion

Our results demonstrate that Wasit province has a high prevalence of Echinococcus spp. in stray dogs. , the G1, G2, G3, G5and G6 genotype was found in Wasit and proved the dominance of E. granulosus s.s. (G1-G3 strains). These findings are crucial for the control and prevention of echinococcosis in the region. Further molecular characterization is essential to better understand the distribution and diversity of Echinococcus spp.

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