Deanship of Graduate Studies

Al-Quds University

Molecular Detection and Genotyping of Hydatid Cysts in Slaughtered Livestock in the West Bank, Palestine

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Dedication

Praise be to God before and after.

I am grateful to God for granting me success in carrying out this thesis.

I dedicate this thesis to my family and many loved ones, especially my beloved mother, who has been a blessing to us over all the years through her endless love, prayers, giving, sacrifices, and Du’a’ for me and my brothers.

I am very thankful and dedicate my thesis to my dear husband and my second family.

For Gaza's children's souls! Peace be upon you.

Wala’a Habes Mohammad Younes

Declaration

I certify that this thesis submitted for the degree of Master is the result of our research, except where otherwise acknowledged, the content of this thesis is the result of work that has been carried out since the date of approval of the research program. That thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution. All ethics procedures and guidelines have been appropriately followed while preparing the thesis.

Signature:

Wala'a Habes Mohammad Younes

Date: 8/1/2025

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For you all…

Thank you from the deepest of my heart.

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Abstract

Background: Despite the widespread occurrence of CE, there is a significant gap in understanding the specific Echinococcus genotypes present in various regions, including the West Bank of Palestine. This lack of knowledge hampers effective disease management and control strategies. Identifying and genotyping the parasites affecting the intermediate hosts is crucial for epidemiological surveillance, Public Health Implications by informing health authorities and policymakers to implement effective control measures based on local epidemiological data. This study aimed to identify and genotype the Echinococcus parasite genotypes causing CE in the West Bank of Palestine, specifically targeting the intermediate hosts (IMHs) such as sheep and goats.

Materials and Methods: A total of 54 cyst tissue samples were collected from livestock in the West Bank, 52 sheep samples from different slaughterhouses from areas known for high CE incidence in the West Bank consisting of hydatid cyst tissues were isolated from infected organs during examinations of the intermediate host, and two are human cysts tissue collected from hospitals after surgery as accidental intermediate hosts. DNA was extracted and amplified by PCR to ensure high-quality genomic DNA for sequencing using the EgG1 *Hae* III sequence (266 bp). The extracted DNA was subjected to NGS using two primers pools, allowing for comprehensive genetic analysis. Targeting all **SNPs** of both mitochondrial genes at once: **COX-1** (Cytochrome c oxidase subunit 1) and **NAD-1** (NADH dehydrogenase subunit 1) for the first time in the present study, which were selected for their variability and are useful for distinguishing between different *Echinococcus* species and genotypes. Finally, using the Variant Call Analysis platform for identifying and genotyping of *Echinococcus* specific sequences among thousands of sequences through online bioinformatics software Galaxy/Europe.

Results: An **EgG1 *Hae* III** sequence of 266 bp was identified in 98% of samples. NAD1 and COX-1 specific fragments were successfully amplified and sequenced through NGS platform from 53 cyst tissue samples. Genotyping analysis was successfully performed in 84% of sequenced samples, revealing that 58% were the G1 genotype and 42% were for the G3 genotype.

Conclusion: This study was to distinguish between *s.s* genotypes G1 and G3 in our isolates based on seven different informative regions together for the first time using NGS with high-quality outputs confirmed the predominance of the G1 genotype while also indicating a significant presence of the G3 genotype among

the intermediate hosts in endemic areas of Palestine. While the sensitivity of NGS is widely acknowledged in the scientific community, our findings emphasize the importance of understanding the local epidemiology of CE. The identification of both G1 and G3 genotypes in this region provides crucial data that can inform future public health strategies and control measures.

Keywords: *E. granulosus*, Intermediate host (IMH), mtDNA, (NGS), Echinococcosis SNPs.

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List of abbreviations

Abbreviation	Full word
AE	Alveolar echinococcosis
Acc. No.	Gene Bank accession number
AD	Anno Domini (after Christ to the present)
BLAST	Basic local alignment search tool
Bp	Base pair
CE	Cystic echinococcosis
CT scan	Computerized tomography
COX-1	Cytochrome c oxidase subunit-1
DNA	Deoxyribonucleic acid
E. canadensis	*Echinococcus canadensis* tapeworm
E. equinus	*Echinococcus equinus* tapeworm
E. felidis	*Echinococcus felidis* tapeworm
E. granulosus	*Echinococcus granulosus* tapeworm
ELISA	Enzyme-linked immunosorbent assay
E. multilocularis	*Echinococcus multilocularis* tapeworm
E. ortleppi	*Echinococcus ortleppi* tapeworm
FGF	Fibroblast growth factor
IMH	Intermediate host
IHA	Indirect hemagglutination
IEP	immunoelectrophoresis
LL	Laminated layer
mtDNA	Mitochondrial DNA
NAD-1	NADH dehydrogenase subunit-1
NGS	Next-generation sequencing
NCBI	The National Center for Biotechnology Information

NTDs	Neglected tropical diseases
NIH	National Institutes of Health
PCR	Polymerase chain reaction
PAIR	Puncture, Aspiration, Injection, Re-aspiration) technique
RT-PCR	reverse transcription polymerase chain reaction
S.L or *s.l*	*Senso lato*
S.S or *s.s*	*Senso stricto*
SNPs	Single nucleotide polymorphisms
WHO	World Health Organization

Chapter One:

General Introduction

1.1 Background:

Echinococcosis or hydatidosis is indeed a serious neglected zoonotic disease that poses significant health risks to humans. It is primarily caused by the larval stage of the *Echinococcus* tapeworm, which can infect humans through various pathways associated with domestic and wild animals. such as canids, wild ungulates (sheep, cattle, and goats). Echinococcosis is still a threat, and it is endemic widely all over the world, especially in rural areas where people have a great interest in livestock breeding, and noticeably in areas with poor public health and hygiene practices(El Berbri et al., 2015). Stray dogs also contribute as one of the epizootiological factors in transmitting the disease. It has become a near-cosmopolitan problem that can cause serious morbidity and death worldwide. Food and agriculture organizations also considered *E. granulosus* and *E. multilocularis* as foodborne parasitic diseases(Inceboz & Inceboz, 2023). The tapeworm *Echinococcus* from the *Taeniidae* family is responsible for the disease affecting mammals. About 2.2% of surgical patients infected with CE have died, and relapse about 6.5% of cases after intervention, which requires a high cost for prolonged recovery time. According to the WHO Foodborne Disease Burden Epidemiology Reference Group (FERG) 2015, echinococcosis is blamed for the death of 19,300 people and around 871,000 disability-adjusted life-years (DALYs) worldwide each year(WHO, 2021). A substantial high annual economic impact caused by cystic echinococcosis, which is estimated at 3 billion USD for treatment and loss compensation among livestock's ranks(WHO, 2011).

Echinococcosis forms a cyst in many organs of the human body, which is, the reason why it's named cystic echinococcosis (CE) most commonly engages the liver in 50 to 70% of most cases which leads to hepatic insufficiency ending with cirrhosis followed by metastasis, and lungs 10-30% leading to dyspnea after cyst enlargement, it also infects the spleen through the portal blood circulation reaching the muscle tissue in late cases(Kırış et al., 2022). The progressive type caused by *echinococcus multilocularis* "alveolar echinococcosis" frame aggregation of small cysts

indicates the appearance of malignant neoplasia, a tumor-like lesion most often in the liver(Gessese, 2020a). Humans become vulnerable to infection by accidental ingestion of the parasite's eggs; the pathogen's transmission is represented in the hand-to-mouth transfer of feces from the infected host into contaminated food or water. After a long incubation period, cysts form and interfere with the body organs' functions. Unfortunately, it's an asymptomatic disease, it is classified as contingent upon its metastasis, which costs high sums for diagnosis and treatment(Casulli et al., 2023). However, early and specific detection helps to relieve the disease's severity and morbidity. Collaboration efforts between veterinarians and health sector workers must be united to control the condition.

Previously, discovering the tapeworm was through intestinal washes of slaughtered animals, followed by developing coproantigen tests, it was a more gene-specific method but had a limited sensitivity, necessitating the need to invent new methods with high specificity and sensitivity(*CDC*, 2024). After that, polymerase chain reaction (PCR) was invented to diagnose the disease and parasite in a specific way(Abbasi et al., 2003). Next-generation sequencing (NGS) recently provides broad prospects for accurate detection and differentiation between pathogens' species. Variation between species affects the clinical presentation of symptoms and the chosen hosts(Budke et al., 2017).

1.2 Epidemiology:

Echinococcosis is widespread worldwide, particularly in areas highly reliant on livestock raising. Species patterns may differ from one region to another depending on the host and environmental conditions. *E. granulosus* is the most common species, which is globally distributed with an incidence of 3 million people annually, estimated by one to two hundred cases per 100,000 individuals in endemic regions, particularly in Mediterranean regions, such as Europe, Africa (east and north), Australia, South America, and Asia. On the contrary, *E. multilocularis* is less popular compared to CE and inhibits the northern hemisphere primarily in the Middle East like Iran, Turkey, Iraq, and Tunisia(Sadjjadi, 2006), also in Europe, and North America with an incidence of 0.03 to 1 case per 100,000 individuals. As for *E. vogeli* and *E. oligarthus*, they reside in South and Central America(Gessese, 2020b).

The World Health Organization (WHO) advocates control procedures for the disease as quickly as possible due to the high incidence rate in humans, which was estimated at 50 per 100,000 population annually in endemic regions. In South America, high incidence prevalence rates were found in livestock slaughterhouses estimated to be 20 to 95 % between carcasses(WHO, 2021).

In Palestine, several studies have revealed the prevalence of CE estimated at 1.5 per 100,000 persons in the Nagab area and around the city of Rahat(Al-Jawabreh et al., 2015). The incidence rate diagnosed in humans during surgery was 1.2 per 100,000 in the West Bank and 1.0 per 100,000 in the Gaza Strip(Al-Jawabreh et al., 2015), who had reported an infection rate of up to 14.3% in dogs. Regarding the IMH, a few information are available about that infection in Palestine, a 9% prevalence of infection was shown in sheep according to Adwan et. al(Abu-Hasan et al., 2002). The disease is still prevalent these days as reported recently in many cases in the Gaza Strip and other Palestinian regions(Shahid et al., 2022; Warasna et al., 2024).

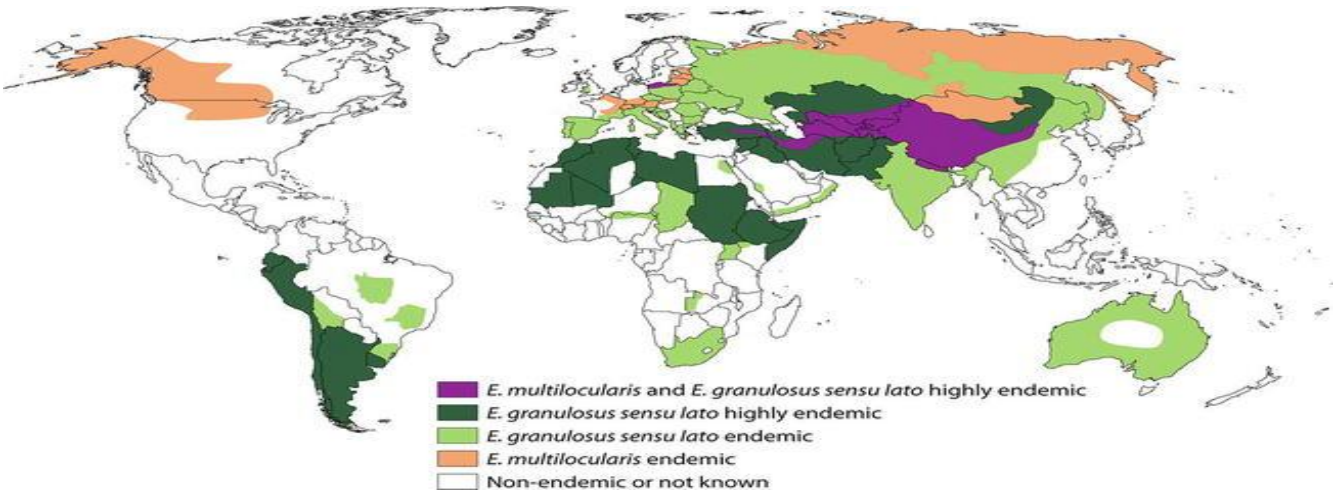


Figure 1.1: *Echinococcus granulosus* and *Echinococcus multilocularis* global distribution(Inceboz & Inceboz, 2023).

1.3 Etiology, species and genotypes:

Echinococcus tapeworm encompasses more than twenty different species, and several genotypes, not all have a pathogenic effect on humans or animals; only four of the species pose significant health concerns for humans, as will be clarified. Four major species are classified based on biochemical markers, genetic features, and specifications, which develop echinococcosis: (1) *E. granulosus*, which induces cystic hydatid disease, is the basic category of many species and genotypes of (a) *E. sensu lato*, which is split into *E. senso stricto* with genotypes **G1 (AB786664)** also known as domestic sheep strain is the most common strain of the disease specifically in the Mediterranean basin regions, **G2 (AJ237633)** known as Tasmanian sheep strain but is no longer a valid genotype, **G3** buffalo strain in Pakistan-India(Bonelli, Dei Giudici, et al., 2021), (b) *E. equinus* **G4 (AF346403)** horse species, (c*) E. ortleppi* **G5 (AB235846)** cattle strain, (d) *E. canadensis* **G6 (AB208063)** camel strain in Sudan, **G7 (AB235847)** pig strain in Poland-Europe, **G8** Cervid strain, and **G9** wildlife species like lion, up to **G10** Eurasian Reindeer strain or Fennoscandian cervid strain(Lavikainen et al., 2006). (2) *E. multilocularis* which causes alveolar echinococcosis, is widespread in the northern hemisphere; it's rare but is the most maleficent than others genotypes: *Taenia hydatigena* **(AB027135)** and *Taenia crassiceps* **(AB033411)**. (3) Polycystic hydatid disease caused by infection with *E. vogeli*; genotype **(M84670)**. (4) And *E. oligarthus* that causes Unicystic hydatid disease, genotype **(M84671)**,(Casulli et al., 2022). Recently, two new species have been discovered, namely *E. shiquicus* which affects mammals, and *E. felidis* in lions of Africa. Although, potential transmission to humans remains unknown(Inceboz & Inceboz, 2023).

1.4 Life cycle and transmission:

The infection occurred through the interaction between the definitive host and the IMH, with an intervention of environmental factors. The cycle begins when an adult tapeworm of *E. granulosus* resides in the intestinal mucosal layer of the definitive host (primarily dogs or other canids), known as the sexual stage of the tapeworm(Díaz et al., 2015), the definitive host becomes infected by ingesting the intermediate host's organs that harboring the cysts of the parasite, the mature adult

tapeworm has pockets full of eggs called proglottids. Once the eggs are released after the proglottids are passed with the feces into the environment, an IMH (herbivores, primarily sheep) ingests feces containing eggs within contaminated food or water, the tapeworm can produce up to 50,000 to 100,000 eggs per proglottid(*CDC*, 2024). After ingestion by an IMH, eggs hatch in the small intestine and release a six-hooked oncosphere (tapeworm embryo-differentiated stage). Those oncospheres migrate through the bloodstream or lymphatic system into various organs after penetration of the intestinal wall of the host, which forms a thick-walled full-filled hydatid cyst in the chosen organ, it could reach a 13cm in diameter length over years, most likely happens in the liver and lung or could be formed in other organs like brain, kidney, spleen also in bones, especially the femur bone where the tapeworm's favorite place as a secondary infection(Leslé et al., 2013). After the enlargement of the hydatid cyst, the protoscolices will be produced. Once the definitive host becomes infected by ingestion of the infected organs of the IMH, protoscolices (the parasite larvae's pathogenic form) evaginate and attach to the intestinal mucosa and develop into the adult stage in about a month to 80 days intervals, completing the life cycle(Moro & Schantz, 2009). Humans are considered aberrant IMHs, they become infected by accidentally ingesting the parasite's eggs from contaminated vegetation or direct contact with contaminated surfaces. Eggs, in turn, will release oncospheres into the intestine and develop a hydatid cyst in various organs. If cyst rupturing has occurred, another cyst will be formed in another site of the body - secondary echinococcosis -, the complexity of this life cycle resulting from the involvement of different hosts varies from one region to another, which in turn, indicates the importance of proper hygiene to control the prevalence and transmission of *Echinococcus* tapeworm and prevent the loss of livestock industry. Once awareness of individuals is raised and the mechanism of disease transmission is understood, they collaborate more easily in controlling the spread of the parasite(Gessese, 2020a).

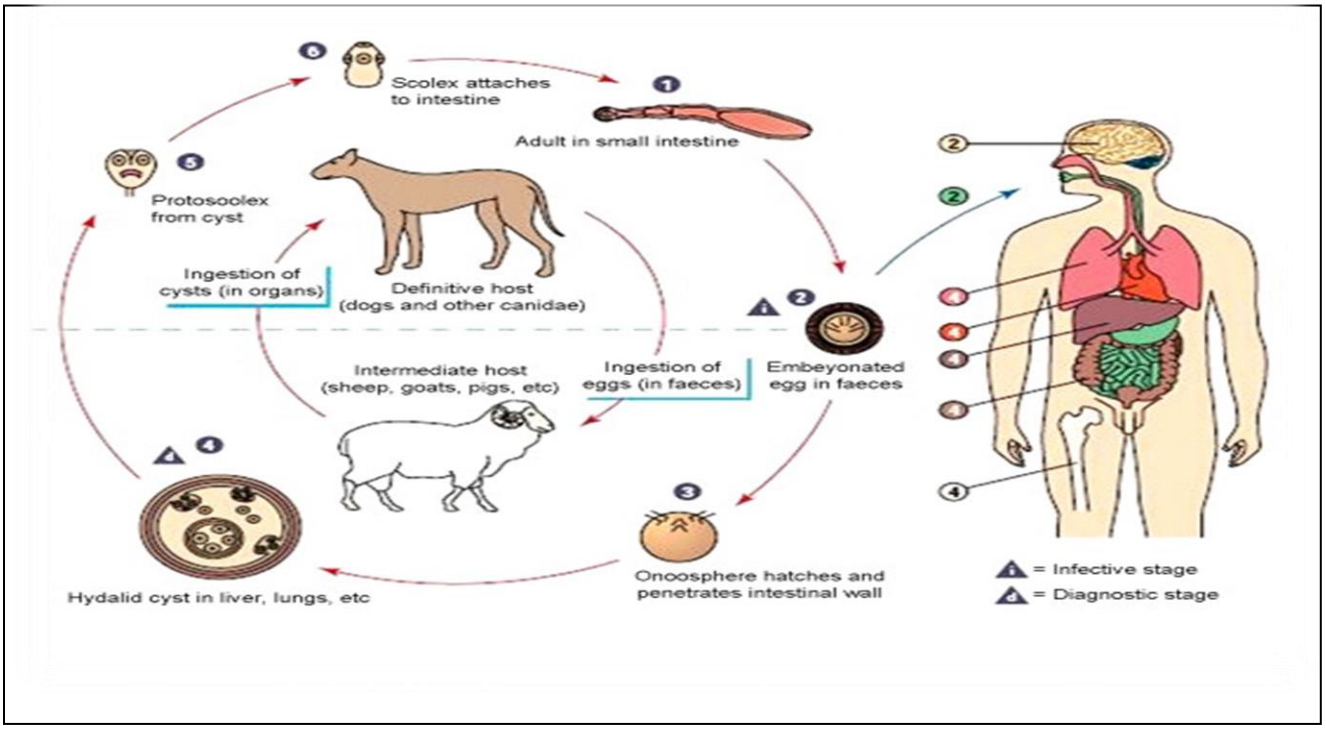


Figure 1.2 The diagram of *Echinococcus* tapeworm life cycle and transmission(Moro & Schantz, 2009).

Hydatid cysts contain fluids surrounded by an outer membrane, the laminated layer (LL), which protects the cyst from any environmental condition and guarantees the survival of the cyst for a very long period. An inner membrane, the germinal epithelium surrounds the cyst's internal component that plays a role in disease invasion. Protoscolices (tapeworm immature head) are located in clusters, penetrating the germinal epithelium, forming the brood capsules as shown in **Figure 1.3**. When the brood capsules are snatched from the inner layer, free hydatid sand will be released into the fluid within the cyst. If the cyst occasionally ruptures exogenously, the hydatid sand will be released, and the parasite will spread all over the host body(Gessese, 2020a).

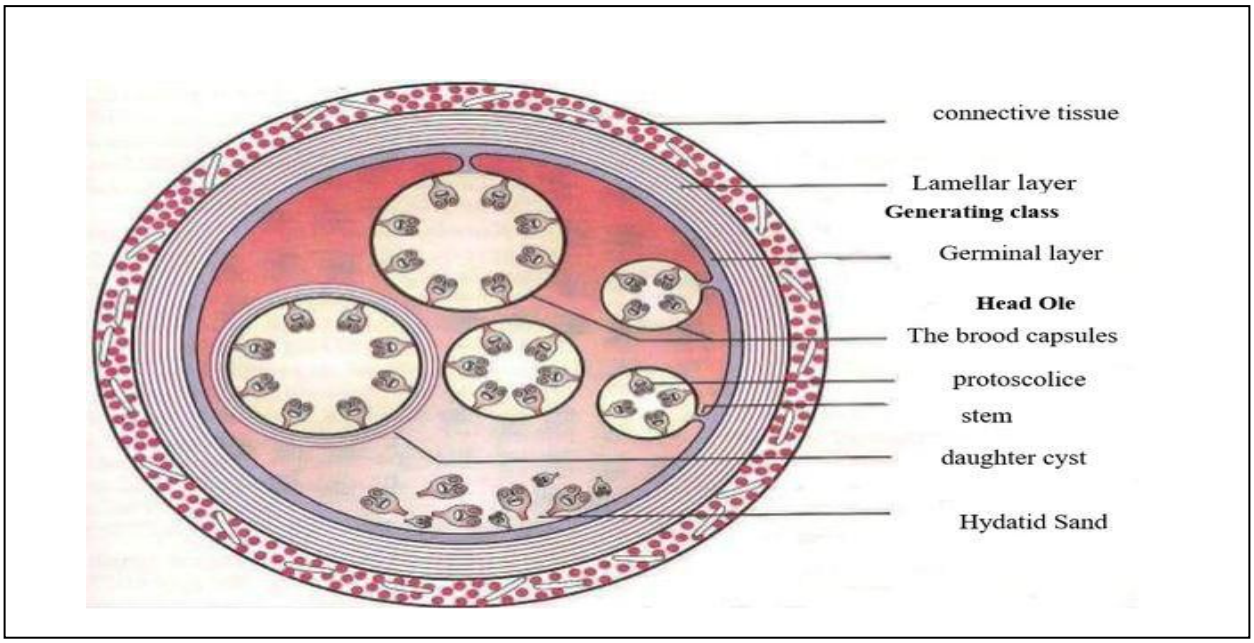


Figure 1.3 A cross-sectional structure of hydatid cyst internal components(Al-Khalidi et al., 2020).

1.5 Diagnosis and genotyping:

It is difficult to predict an asymptomatic infection depending on the traditionally used methods to identify and genotype the pathogen species. Some serious clinical symptoms induced by a cyst highlight the need for additional test methods such as serological tests (ELISA, IHA, and western blot) and imaging methods (ultrasound, CT, or MRI) which were all used in the first place to detect the cause of a cyst because of its inexpensive and fast for screening. However, they are limited in identifying a specific pathogen or even distinguishing between its species and genotypes; they could overlap with other diseases with similar symptoms, leading to false positive results(McManus et al., 2012; Tamarozzi et al., 2021). Molecular diagnostic assays were also used to detect the parasite such as simple PCR which is a highly sensitive and specific method than previously in detecting pathogens, PCR can distinguish between *E. granulosus* and *E. multilocularis*, however, it is relatively expensive compared to its throughput, low availability in endemic areas, false negative or positive samples are a common problem in poor-quality or contaminated samples. Recent research has depended on invented methods to overcome the usual limitations of previous and some recent molecular methods like multiplex PCR, real-time PCR for viability assessment, and LAMP (Loop-Mediated Isothermal Amplification)(Knapp et al., 2023),

despite their speed, lower cost, improved sensitivity and specificity to others, and their concurrent detection of multiple pathogens or genotypes in a single test, they are also technically demand validated kits, many time-consuming steps, and limited sensitivity in detection all samples(R. J. Zhang et al., 2022). More recently, next-generation sequencing (NGS) has overcome all the previous molecular techniques due to its high-quality throughputs through massive data deep sequencing in a parallel mechanism with extremely high sensitivity and specificity within a short period. It presents a precise, detailed genotyping of the parasite and facilitates variations and mutations identification, it is ideal for epidemiological studies, and not as expensive as many people think, NGS offers a sharing run for multiple samples which lowers the cost of sequencing with high-quality millions of reads compared to Sangar sequencing which give only one read with high cost(Chiu & Miller, 2019). Conversely, other invented sequencing and genotyping methods like microsatellite genotyping need previously known databases for comparison, intensive work, and advanced genetic analysis mechanisms with relatively high cost. Other methods may require additional equipment for visualizing data like gel electrophoresis as in Restriction Fragment Length Polymorphism (RFLP) which was widely used for sequencing because it gives detailed information at the genetic level but only in large amounts of DNA which consumes time and money and may result in Cross-reactions between similar species(Chaâbane-Banaoues et al., 2016). Single-nucleotide polymorphism (SNP) Genotyping is also useful for identifying different strains and detecting their genetic diversity. It is also an expensive technique that requires complicated equipment and bioinformatics tools. However, when combined with NGS, a potent and distinctive technique will facilitate epidemiological studies using bioinformatics tools like Galaxy platform(Huang et al., 2016). The necessity to invent new methods, such as NGS, arises from their benefits in the detection and genotyping of Echinococcus species.

1.6 Treatment:

The treatment of domestic animals is recommended; some anthelmintic drugs such as praziquantel among others, have now shown high markers of effectiveness in the treatment of infected animals. Studying the genetic diversity of the parasite has serious implications for the development of treatment pathways, drug or vaccine designs, and diagnostic methods(Kern et al., 2017). WHO had classified the cyst-specific-stage management approach by ultrasound, especially in the case of hepatic hydatid cyst(Bell & Venkatesh, 2013). To be clear, some cases need only therapy treatment, but most cases need surgical intervention with sterilization methods according to species malignancy and stage, some may need “wait and watch” approach or combination as in AE infection which silently progresses a liver disease that may metastasize to another location which in most cases require an immediate surgical intervention, meanwhile CE may need therapeutic drugs alone at the first place and may not need a surgery, or may need both at late-stage cases(Kern et al., 2017).

Surgical removal of cysts is the best choice of treatment, but recurrence is common; the combination of prolonged drug therapy will be needed. Four summarized approaches are followed in the treatment of CE: (1) percutaneous treatment of the hydatid cysts with the PAIR (Puncture, Aspiration, Injection, Re-aspiration) technique; (2) surgery (the first choice), (3) anti-infective drug treatment, the anthelmintic drugs like Mebendazole, Albendazole, and Prazionlantel, they could be used before the surgery to prevent infection metastasis or after to get rid of any remnants of the parasite. The last choice is (4) “watch and wait”(Vuitton & Wen, 2007). The choice of

treatment should primarily be based on ultrasound images of the cyst, following a stage-specific approach, which also depends on the medical infrastructure and human resources available before performing the surgery.

1.7 Objectives and study significance:

The main aim of this study is to identify and genotype the *E. granulosus* within the IMH cyst tissue using molecular methods, targeting repeat sequences (EgG1 *Hae* III) 266 bp from cyst tissue of sheep from different slaughterhouses and human cyst tissue from hospitals after surgery in the West Bank of Palestine. Furthermore, to investigate the genetic diversity and genotyping of the parasite by developing methods like next-generation sequencing based on targeting the mtDNA COX-1 and NAD-1 SNPs, to identify the most predominant species and genotypes of *E. granulosus* that infect the IMH in Palestine. The findings of this study will rebound to the benefit of society, considering that humans are highly dependent on the livestock industry and feeding, not enough information about the disease in IMH has been established yet, phylogenetic study is an urgent requirement.

Chapter Two:

Literature Review

2.1 A brief history of Echinococcosis:

Before the 19th century AD, the *e. granulosis*'s life cycle was unknown, although it has been a major cause of death since then. After that, many researchers determined the prevalence and the etiology of that disease, which interferes with controlling the disease burden more efficiently than before by proper intervention criteria based on molecular and phylogenetic studies. New Zealand and Tasmania succeeded in converting CE magnitude from epidemic into extinction status(Gemmell, 1990). In 1801, an Italian researcher, Rudolphi discovered the genus of *echinococcus*. After a while in the 19th century, a French scientist Deve, clarified the structure of the echinococcosis cyst and the possibility of the parasite metastasizing and forming another cyst in a different site of the body, a "secondary echinococcosis". In the middle of the same century, alveolar echinococcosis (AE) was classified as an independent entity rather than cystic echinococcosis (CE), and it took many years to prove that the cause of AE and CE is not the same species of the parasite *echinococcus*(Dakkak, 2010). For now, WHO classified Echinococcosis as one of the 17 global neglected tropical diseases (NTDs) with two main species of medical interest, *E. granulosus* and *E. multilocularis*(Higuita et al., 2016). *Echinococcus* is associated with carnivores in nature which represent the definitive hosts as canines that carry the adult cestode in their small intestine, and with herbivores as the intermediate host as sheep carrying the larval stage of the parasite(Higuita et al., 2016). The eggs released from infective dogs harboring oncospheres which penetrate the intestinal wall of the intermediate host after ingestion of the infected eggs and transform into hydatid cysts in various organs. Humans may ingest the *Taenia* eggs accidentally through contaminated soil, food, or water(*Echinococcosis*, 2023). The major pathogenic species distributed worldwide is the *E. granulosus* which is responsible for the most human cases of echinococcosis in endemic areas that are mainly dependent on livestock breeding for food and industry(Lia & Stella, 1997). *E. multilocularis* is considered a more aggressive and complicated infection that may form tumor-like lesions in the liver, as recognized by the German pathologist Virchow in 1855(Llterat, 1855), he revealed the real cause of those tumor lesions was *E. multilocularis,* which causes alveolar echinococcosis. One hundred years later, scientists could differentiate between the *echinococcus* species(Ammann & Eckert, 1996).

2.2 Hydatidosis prevalence:

Dakkak and his colleagues also represented the epidemiology of hydatidosis in detail all over the Mediterranean regions, confirming that *E. granulosus* and its genotypes are the most common species responsible for echinococcosis, explaining the factors that influence the spread of that parasite in the Mediterranean regions, suggesting by data the growing prevalence in some regions more than others due to high morbidity rates in those areas, which increase the economic burdens, like in Turkey, Greece, North Africa, and some regions of Spain(Dakkak, 2010). A systematic review of the global Prevalence of Echinococcosis in Goats was conducted by Yan and his colleagues in 2022, which was performed on 108,197 samples, finding that echinococcosis was wide-ranging in goats based on many factors like age and climate. The total prevalence was to be 10.85% overall(Yan et al., 2022). In 2016, Founta and his colleagues revealed a high prevalence of hydatidosis in sheep more than in other slaughtered species for human consumption in Northern Greece(Founta et al., 2016). In the west of Saudi Arabia, specifically in a huge abattoir in Makkah, the prevalence of hydatidosis in goats was 0.23% through a cross-sectional study during 2018, focused on heart and spleen mainly(Alsulami, 2019). Riyadh city, the capital of Saudi Arabia, had its share of that infection; 12,569 Sawakny sheep were inspected, resulting in an overall prevalence of 1.06%, especially in the liver and lungs of the sheep(Almalki et al., 2017). One of the agricultural countries conducted a cross-sectional study through a survey based on PCR, amplifying the two mitochondrial genetic markers NAD-1 and COX-1 genes; the prevalence of CE in randomly examined 2833 animals was 9% in Khyber Pakhtunkhwa, Pakistan(Khan et al., 2021). In the Republic of China, a meta-analysis study was conducted to study the prevalence of hydatidosis in bovines as an intermediate host for the first time between the period of 2000 to 2021, the infection was estimated to be 17.27% after analysis of published data(Fan et al., 2022). As in the Mediterranean regions which recorded a high prevalence of CE like in Turkey, with an overall prevalence of 3.93%, considering many factors like climate, months, and gender. Ending with economic losses of 56,434 USD due to hydatidosis in the livers of the intermediate hosts in Konya, Turkey(Küçükyağlıoğlu & Uslu, 2022). A prevalence of hydatidosis, estimated at 13.9% in Iran through a meta-regression analysis, summarized 31 published articles, analyzing the increase in sample size and publication year by ($P < 0.05$)(Vaisi-Raygani et al., 2021).

In Palestine, particularly in humans, A seroprevalence study carried out on Bedouin and Jewish people in 2002 showed a percentage of 0.68% and 0.5% respectively which related to a high incidence rate of the disease among livestock slaughtered in the Negev area in southern Israel by using enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G, indirect haemagglutination (IHA) & immunoelectrophoresis (IEP)(Youngster et al., 2002). In 2002, a Sero-epidemiological study was conducted to study the surgical incidence of echinococcosis in the West Bank of Palestine, finding an overall mean annual surgical incidence (MASI) estimated at 3.1 per 100,000 in Hebron, Jericho and Bethlehem Governorates. Yata town at Hebron recorded the highest MASI, which was estimated at 16.8 per 100,000 due to high population density(Abu-Hasan et al., 2002). Al-Jawabreh and his colleagues conducted a cross-sectional study we estimated on the definitive host by Copro-PCR method, using fecal samples of 93 domestic dogs to reveal the incidence of *E. granulosus* targeting *EgG1 Hae III* sequence, also revision of medical archives in different 9 main hospitals in Palestine to determine incidence of infection in humans after surgery, genotyping and sequencing of results, referring to the mitochondrial gene cytochrome c oxidase subunit-1 (COX-1), finding represented as mentioned

before as follows: 1.2 per 100,000 and 1.0 per 100,000 in the West Bank and Gaza Strip respectively. For domestic dogs, the infection rate is estimated at 18% of samples, leading to a high prevalence of the infection in dogs in Palestine(Al-Jawabreh et al., 2015). Followed by a new research for the same authors to investigate the burden of cystic echinococcosis between 2010 and 2015, they found that the incidence of CE in Bethlehem and Al-Khalil was 3.7 and 4.2 per 100,000 over a 6-year average by statistical analysis, Al-Khalil province has reported the highest surgical incidence of cystic echinococcosis (CE) in Palestine, estimated at 2.1 per 100,000 cases, this was the first to confirm by molecular methods the human causative species of echinococcosis infection in Palestine, which was the *E. granulosus sensu stricto* (*s.s*) species, representing in 11 samples registered the **G1** genotype, and two samples revealed the presence of the **G3** genotype. Although **G1** genotype was the predominant genotype in the rural areas of Palestine. They also found that cystic echinococcosis is spread among adolescents, people between the ages of 10 to 20 years old, which indicates that CE infection is localized in humans from an early stage in life and remains for years before presenting any clinical signs, a long latency period(Al-Jawabreh et al., 2017). Hamarsheh and his colleagues compared the epidemiology of 12 parasitic infection included hydatid disease among others in Palestine from 2008 to 2017 between the West Bank and Gaza Strip. The average annual incidence rate of hydatidosis in the West Bank was 8.39% per 100,000 and 3.36% per 100,000 in Gaza Strip, estimated on a total of 137,106 cases reported by the Palestinian Ministry of Health and surveys were conducted in Gaza Strip(Hamarsheh & Amro, 2020). A recent study in the Gaza Strip - Palestine emphasizes the importance of raising livestock awareness which should be directed to the public and health sectors, through several recommendations based on potential risk factors and genetic study of the parasite within the definitive and the intermediate host to improve the control of the disease prevalence. Some of the recommendations were presented by controlling the spreading of free-roaming dogs due to high prevalence in overpopulated regions, educating the population about the transmission of the disease to take the proper interventions, and informing the health sector workers about the incidence of the disease to deal with. Also, the Butchers have a huge responsibility in controlling the spread of the disease by getting rid of the IMH-infected organs away from free-roaming dogs and committing to the correct slaughter instructions in their respective places under the supervision of the municipality(Al-Hindi et al., 2023).

A few studies have addressed CE in the intermediate host in Palestine. According to echinococcosis history in Palestine by 1977, Matossian and his colleagues showed the prevalence of CE in the IMH varied between 0.02% to 12% in Tel-Aviv and Beersheba, respectively(Matossian et al., 1977). By the 1990s, Yirka-Northern Israel recorded a high incidence rate of cysts in a local abattoir, with about 10% of the sheep infected only in one month according to a survey(Furth et al., 1994). According to Hammed and his colleagues, a prevalence study carried out on some slaughterhouses in the West Bank of Palestine, revealed the relation between the IMH and the susceptibility to the disease through morphologic features of the cyst and the risk factors related, as the IMH age and some else, the total prevalence of hydatid cyst was 9 % of 1000 sheep carcasses(Hammed et al., 2009). No reliable formal records regarding the CE genotypes in the IMH in Palestine have been disclosed yet by the Palestinian Ministry of Health or Agriculture.

2.3 *Echinococcus* genotype - host's organ relation:

Many researchers investigated the host predominant genotype in different countries which is beneficial for detecting in which organ the parasite is localized to facilitate the diagnosis of each species and to give the proper treatment as soon as possible(Hajjafari et al., 2024). G1 cysts are usually well-studied, presenting several biological behaviors based on the current stage (active, transitional, or inactive). In turn, G3 cysts might show uneven growth rates and immune evasion strategies, although comprehensive comparative studies are limited; this may refer to variations in nucleic acid sequences that are expressed in phenotypic traits that influence several aspects, including the life cycle pattern, host specificity, and development rate. These differences also impact pathogenicity, antigenicity, and the sensitivity to chemotherapeutic agents. Additionally, they affect the transmission dynamics, epidemiology, and control strategies of cystic hydatid disease(Thompson & McManus, 2002). A periodic checkup may be necessary in endemic areas that are interested in livestock breeding, taking into consideration the genotype-host correlation. For example, pastoral and poor rural communities that are common in bovine breeding should construct medical examination programs to check on the G3 genotype in people who are in direct contact with domestic animals, and so for the G1 genotype which is correlated to sheep breeding(Bonelli, Dei Giudici, et al., 2021). Every genotype of the *echinococcus* species is correlated with a special host as clarified before, but some of them may infect another host, which may affect the treatment plan of the disease(Alvarez Rojas et al., 2014). McManus and his colleagues explained how one genotype could affect several hosts. He mentioned an example of the G1 genotype, which is correlated to sheep strain, but it can also infect other cattles in a sterile manner, forming a sterile cyst that could go away without treatment(McManus & Thompson, 2003).

The invasion process of a specific genotype to affect a particular organ involves immunological and biological mechanisms represented in the parasite's ability to manipulate the host's immune response(Hoberg, 2002). *Echinococcus* larvae excrete substances that let the parasite easily invade the host's immune system, such as cytokines and chemokines which suppress the inflammatory responses and affect the regulatory T-cells, therefore allowing the parasite to survive in the host tissues(Gottstein & Hemphill, 2008). That mechanism could modulate the immune system within specific organs, which is determined by the surface expression of molecules on the parasite that interact with receptors in target organs, that can explain why is liver frequently targeted by many genotypes, that’s because of its role in filtering blood from whole the body, providing a conducive environment for the parasite's establishment through exploiting specific receptors and cellular pathways in host tissues(Gottstein et al., 2017). Some genotypes may express surface proteins that mimic host molecules, facilitating attachment and invasion of particular organs(W. Zhang et al., 2003). Genetic differences among *Echinococcus* genotypes also influence their ability to infect specific organs, with variations in gene expression affecting the parasite's capacity to adapt to the host environment and resist immune attacks(Alvarez Rojas et al., 2014). Environmental and host factors, such as the host's immune status, age, and genetic background, can also impact organ-specific targeting, with immunocompromised individuals being more susceptible to infections in multiple organs(Harriott et al., 2019).

Even though species differ according to the host and environmental conditions, that investigation will assist in quick and efficient intervention for each case. For example, Ohiolei and his colleagues have revealed that *E. granulosus sensu stricto* (**G1**, **G3**) genotypes are found in sheep’s liver and

E. canadensis (**G7**) is found in the liver of pigs. Meanwhile, *E. canadensis* (**G6**) and *E. ortleppi* (**G5**) are predilected in the lungs of camels and cattle respectively(Ohiolei et al., 2022).

The most virulent *echinococcus*; *E. multilocularis*, is found most often in the liver, they found the reason why it selects that organ specifically, refers to the metacestodes of the tapeworm which can demonstrate a homologous fibroblast growth factor (FGF) receptor family, mimic to the human FGF receptor in the liver that helps in proliferation and migration of cells in case of infection(Ohiolei et al., 2022).

2.4 Diagnostic and Genotyping Methods of Echinococcosis past and present:

Several traditional methods were used to detect the presence of the *Echinococcus* parasite and its species. Some of these methods lacked specificity or sensitivity for a specific genus to confirm the diagnosis of the disease. Several diseases could cause cyst formation in different organs of the human body, but not all are caused by the same pathogen(Vachha et al., 2011). Some other types of nematodes, like *Capillaria hepatica* and *Fasciola hepatica*, can interfere with *echinococcus* species in forming cysts in the human body, especially in the liver(Kaya et al., 2011, Miller et al., 2020). Molecular diagnosis is crucial to confirm tangibly that our cyst was formed by the intended parasite, *Echinococcus*. Recently, approaches attempted to detect the epidemiologic pattern of the parasite, as well as to improve treatment management and control procedures by the invention of new highly specific and sensitive methods to facilitate the capture of variants of specific genomes and species differentiation(Deplazes et al., 2017). Despite the benefits of the traditional previous methods, they lack the efficient analysis of a huge number of sequences, they are limited, less sensitive, and less specific in many cases which need further investigation methods to clarify the specificity of the genome. A newly developed system that overcomes these problems has arrived to obtain a deep study of any particular disease quantitatively, the invention of next-generation sequencing (NGS) gives precise, detailed knowledge about a pathogen or disease epidemiology, and proves its worth in overcoming all of the analysis issues by performing fast and efficient parallel deep sequencing for huge numbers of genomic data or even the whole genome in a relatively short period, without the need for a database or previous knowledge in detecting variants within the targeted genome, and affordable at the same time(Rao et al., 2020).

Historical diagnostic methods primarily relied on clinical symptoms such as abdominal pain, nausea, and organ enlargement, combined with physical examinations to detect cysts, particularly in endemic areas, but were not specific(Eckert & Deplazes, 2004). Early imaging techniques like X-rays were used to identify cysts. However, X-rays provided limited information about calcified and non-sterile cysts and lacked the detail needed for comprehensive assessment. Ultrasound, although non-invasive and cost-effective, can sometimes be operator-dependent, leading to variability in results(Badwaik et al., 2024), but the introduction of ultrasound in the mid-20th century marked a major advancement by allowing the detection of cystic structures in the liver and other infected organs(Eckert & Deplazes, 2004). CT and MRI scans enhanced the detection by providing detailed images for better assessment. While they represent detailed visualization, they are more expensive and not always readily available in resource-limited settings(Kui et al., 2021).

Serological tests also have been widely used in diagnosis, with the early Casoni skin test becoming obsolete due to high rates of false positives. More reliable methods like ELISA and indirect

hemagglutination were developed to detect specific antibodies against *Echinococcus* antigens. ELISA is sensitive and widely used, but it can sometimes cross-react with antibodies from other parasitic infections, leading to false positives(Ghorbanpoor et al., 2006). Modern advancements include Western blotting, which provides higher specificity, based on the use of recombinant antigens to reduce cross-reactivity issues. However, these tests can be more expensive and technically demanding(W. Zhang & McManus, 2006).

Genotyping methods have advanced significantly from initial morphological identification, which lacked precision, to molecular techniques such as PCR-based methods. PCR is highly sensitive and specific, enabling the differentiation of species, but it requires technical expertise and specialized equipment, which can be a limitation in some settings(Jenkins et al., 2005). Restriction fragment length polymorphism (RFLP) provides good differentiation of genetic variants but is labor-intensive and less sensitive than PCR(Bowles et al., 1992). Mitochondrial DNA sequencing offers higher resolution in identifying genetic variations, but is costlier and more time-consuming(Nakao et al., 2013).

Next-generation sequencing (NGS) features are represented as a powerful tool for genotyping, offering several advantages over traditional methods. NGS allows for rapid and comprehensive analysis of entire genomes, providing detailed insights into genetic variations and enabling the identification of novel strains(McManus et al., 2003), because of that, we can use it to investigate the small fragments for the detection of specific SNPs of a particular genome, followed by software programs to analyze the NGS outcomes.

Next Generation Sequencing (NGS) and Sanger Sequencing are two pivotal methods for DNA sequencing, each with distinct characteristics. NGS refers to a collection of advanced sequencing technologies that enable the simultaneous sequencing of millions of DNA fragments, allowing for high-throughput sequencing that can analyze entire genomes rapidly and cost-effectively. One of its advantages includes its capability to produce high throughputs, it can sequence millions of fragments at once, significantly reducing the cost per base of sequencing(Shendure et al., 2017). This versatility allows NGS to be applied in several fields, such as whole-genome sequencing, targeted sequencing, RNA sequencing (RNA-seq), and epigenomics(Satam et al., 2023). Furthermore, NGS is skilled at detecting a wide range of genetic variants, including single-nucleotide polymorphisms (SNPs), insertions, deletions, and structural variants, which are essential for understanding genetic diseases(Koboldt et al., 2013). However, NGS also has its drawbacks. The sheer volume of data generated can be overwhelming, necessitating durable computational resources and bioinformatics expertise for data analysis and interpretation(Mardis, 2008). Additionally, NGS can exhibit higher error rates than Sanger sequencing, particularly in homo-polymorphic regions(Cheng et al., 2023), and typically produces shorter reads, complicating the assembly of genomes and analysis of repetitive regions(Mardis, 2008). The initial setup costs for NGS equipment can also be high, although the per-sample cost decreases significantly in large-scale applications(Shendure et al., 2017). Another advantage of NGS, that there is no need for conventional visualizing methods, such as exposure to UV light. NGS sequencing was performed by NovaSeq 6000 device (Illumina®, USA) which can read millions of short DNA fragments per sample with a specificity of 10,000 reads for each nucleotide which indicates the stability of NGS, Illumina system follows a methodology of sequencing by synthesis (SBS), this method depends on a unique florescent signal emission from special emitter bases, resulting in nucleic acid chain consists of florescent marked bases that later will be analyzed(Moti, 2022). See **figures 2.1** and

2.2, presenting NGS's data processing and then visualizing by Galaxy.

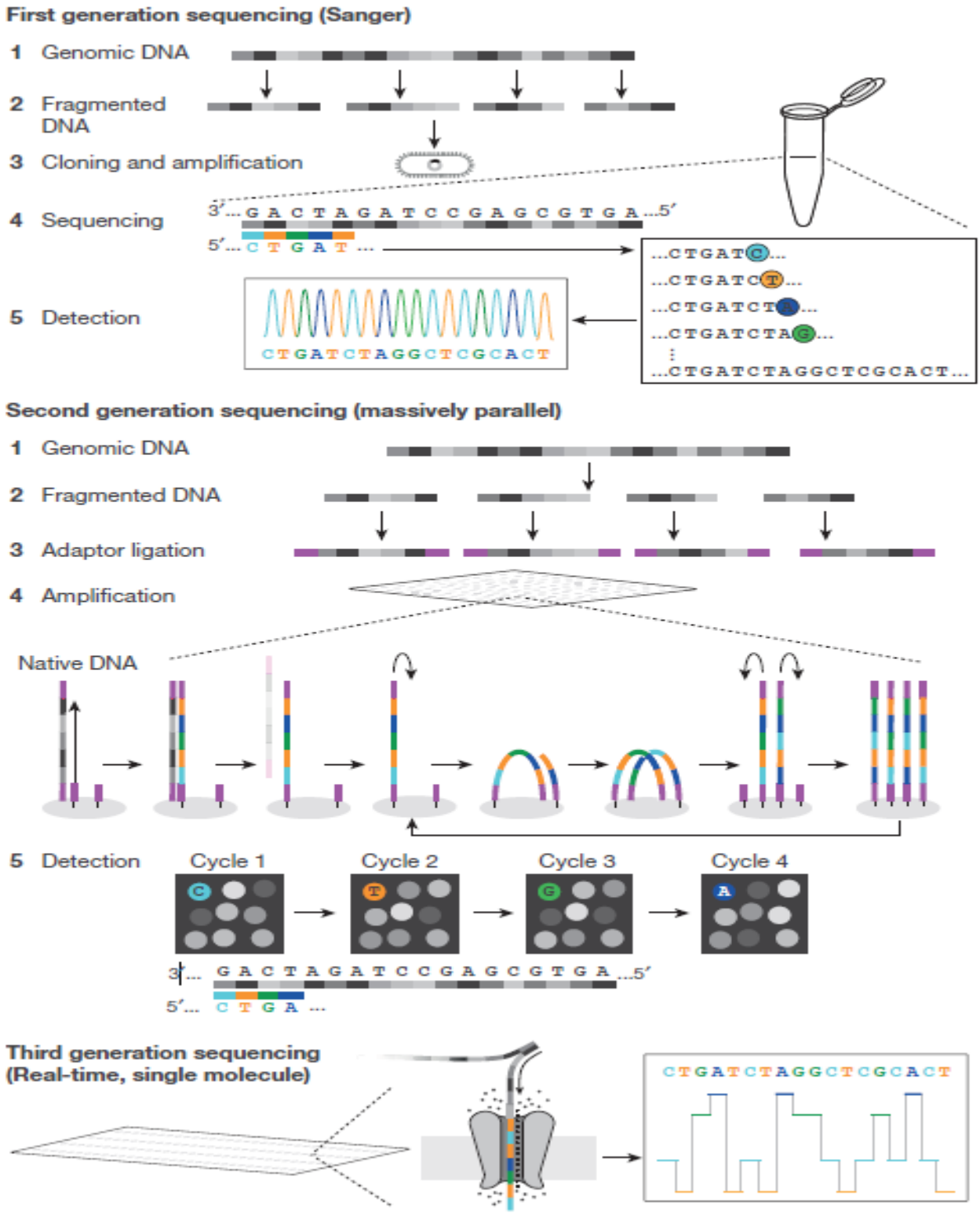


Figure 2.1: Schematic examples of first, second and third generation sequencing. Second-generation sequencing is referred to as next-generation sequencing (NGS)(Shendure et al., 2017).

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	Map_with_BWA_ME
31_NAD_I	1	.	T	.	.	.	DP=250;F	GT	./.
31_NAD_I	2	.	T	.	.	.	DP=251;F	GT	./.
31_NAD_I	3	.	T	.	.	.	DP=252;F	GT	./.
31_NAD_I	4	.	G	.	.	.	DP=253;F	GT	./.
31_NAD_I	5	.	T	.	.	.	DP=254;F	GT	./.
31_NAD_I	6	.	T	.	.	.	DP=255;F	GT	./.
31_NAD_I	7	.	A	.	.	.	DP=256;F	GT	./.
31_NAD_I	8	.	G	.	.	.	DP=257;F	GT	./.
31_NAD_I	9	.	G	.	.	.	DP=258;F	GT	./.
31_NAD_I	10	.	A	.	.	.	DP=259;F	GT	./.
31_NAD_I	11	.	T	.	.	.	DP=260;F	GT	./.
31_NAD_I	12	.	T	.	.	.	DP=261;F	GT	./.
31_NAD_I	13	.	A	.	.	.	DP=262;F	GT	./.
31_NAD_I	14	.	T	.	.	.	DP=262;F	GT	./.
31_NAD_I	15	.	G	.	.	.	DP=263;F	GT	./.
31_NAD_I	16	.	G	.	.	.	DP=264;F	GT	./.
31_NAD_I	17	.	G	.	.	.	DP=264;F	GT	./.
31_NAD_I	18	.	G	.	.	.	DP=265;F	GT	./.
31_NAD_I	19	.	G	.	.	.	DP=266;F	GT	./.
31_NAD_I	20	.	T	.	.	.	DP=267;F	GT	./.
31_NAD_I	21	.	G	.	.	.	DP=268;F	GT	./.
31_NAD_I	22	.	T	.	.	.	DP=269;F	GT	./.
31_NAD_I	23	.	T	.	.	.	DP=270;F	GT	./.
31_NAD_I	24	.	G	.	.	.	DP=271;F	GT	./.
31_NAD_I	25	.	G	.	.	.	DP=272;F	GT	./.

Figure 2.2: An example of visualizing one sample of our isolates processed by NGS and then analyzed through Galaxy.edu

In contrast, Sanger Sequencing, developed by Frederick Sanger in the 1970s, is known for its accuracy and reliability. It uses a method called chain termination, where dideoxynucleotides (ddNTPs) are incorporated into a growing DNA strand, halting synthesis and allowing for the determination of the sequence based on fragment length(Sanger et al., 1977). The primary advantages of Sanger sequencing include its high accuracy, making it the gold standard for confirming variants identified by NGS and for sequencing smaller regions of interest(Schneider et al., 2012). Sanger sequencing typically produces longer read lengths compared to NGS, which aids in resolving complex genomic regions and repetitive sequences.

The table below (**Table 2.1**) summarizes traditional and recent methods, with their main limitations(W. Liu et al., 2014, Abbasi et al., 2003, Gonlugur et al., 2005, Sharbatkhori et al., 2009, Kırış et al., 2022, C. N. Liu et al., 2015, Rao et al., 2020).

Table 2.1: Discriminations between diagnostic and genotyping assays past and present.

	Traditional	Recent	Limitations	References
Diagnostic methods				
1. Imaging methods (US/ CT/ MRI)	Widely used	Non-invasive, still in use as an initial diagnosis of cyst	Not enough for a specific diagnosis	(W. Liu et al., 2014)
2. Serological methods (ELISA, Western Blot, antigen detection, IHA)	Cheap and fast, detection of antibodies/antigen	Widely used tests, fine specificity	Limited sensitivity, cross-reactivity, need large DNA quantities	(Abbasi et al., 2003), (Gonlugur et al., 2005).
3. Molecular diagnosis (PCR, RT-PCR, qPCR, LAMP)	Highly specific and sensitive	Not enough for species and genotype differentiation	Advanced equipment need, may not be validated	(Sharbatkhori et al., 2009)
Genotyping methods				
1. DNA sequencing	RFLP	mitochondrial markers analysis, precise and highly sensitive	Qualitative, time-consuming and low success rate	(Kırış et al., 2022).
2. PCR based methods	PCR, RFLP	Multiplex PCR, NGS, Cox-1, ITS region sequencing (particularly for genotyping)	Requires bioinformatic tools for analysis, not available in many endemic regions	(C. N. Liu et al., 2015)
3. Microsatellite and SNP analysis	RFLP, PCR	NGS, Good-scale differentiation for strains and genotyping	Requires bioinformatic tools for analysis, not available in many endemic regions	(Rao et al., 2020).

Chapter Three:

Materials and Methods

3.1 Ethical approval:

This study was approved by the Research Ethics Committee at Al-Quds University, and verbal approval was obtained from slaughterhouse workers or animal owners.

3.2 Sample collection and storage:

A total of 80 tissue samples were kindly provided from a previous prospective study by Al-Jawabreh and his colleagues for this research from different slaughterhouses in Palestine between 2014 and 2018, summarized in **Table 3.1**. The cysts were transferred to the lab from the remaining offal after slaughtering the animal for food, and the last two samples were human cyst tissue, picked up after surgery. The samples were stored at -20°C at Al-Quds University molecular lab for later molecular and genetic tests. Every sample was properly coded and processed to complete the study's objectives. International DNA samples of *E. granulosus* genotypes were kindly provided by The Epidemiology Collaborating Centre-Detection, and Control of Cystic and Alveolar Echinococcosis (in humans and animals) of the World Health Organization, Higher Institute of Health, Rome, Italy, as reference sequences of *E. granulosus* genes (G1, G3, G4, G5, G6/7, and G10)

Table 3.1.a: Collected cyst tissue samples details:

sample number	type	Animal	tissue origin	organ site	isolation year	region	Gender	Age	Cyst size
EI 1	tissue	cow	Lung	Cyst Tissue	2017	Al-Khalil	Female	3	
EI 2	tissue	cow	Lung	Cyst Tissue	2017	Al-Khalil	Female	4	

Table 3.1.b: Collected cyst tissue samples details:

EI 3	tissue	Goat	liver, omentum	Cyst Tissue	2017	Ariha	Female	2	
EI 4	tissue	Goat	Liver	Cyst Tissue	2017	Al-Khalil	Male	1.25	
EI 5	tissue	Goat	Liver	Cyst Tissue	2017	Al-Khalil	Male	1.25	
EI 6	tissue	Goat	Liver	Cyst Tissue	2017	Ariha	Female	4	
EI 7	tissue	sheep	Liver	Cyst Tissue	2017	Nablus	ND	<1	
EI 8	tissue	sheep	Liver	Cyst Tissue	2017	Nablus	ND	<1	
EI 9	tissue	sheep	Liver	Cyst Tissue	2017	Nablus	ND	<1	
EI 10	tissue	sheep	Liver	Cyst Tissue	2017	Nablus	ND	<1	
EI 11	tissue	Sheep	Liver	Cyst Tissue	2017	Al-Khalil	ND	<1	
EI 13	tissue	Goat	Liver	Cyst Tissue	2017	Al-Khalil-Yatta	ND	<1	0.5x0.5
EI 14	tissue	Sheep	Liver	Cyst Tissue	2017	Al-Khalil-Yatta	ND	<1	5.5x5.5
EI 15	tissue	Goat	Liver	Cyst Tissue	2017	Al-Khalil-Yatta	ND	<1	2x2
EI 16	tissue	Goat	Liver	Cyst Tissue	2017	Al-Khalil-Yatta	ND	<1	2x2
EI 17	tissue	sheep	Liver	Cyst Tissue	2017	Jenin	Male	0.5	3x3
EI 18	tissue	Sheep	Liver	Cyst Tissue	2017	Nablus	ND	<1	2x2.5/1.5x1.5
EI 19	tissue	Sheep	Omentum	Cyst Tissue	2017	Nablus	ND	<1	2x1,5/2x2
EI 20	tissue	Sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	0.66	2x1.5
EI 21	tissue	Sheep	Omentum	Cyst Tissue	2017	Tulkarem	Female	1	2x2.5/2x2.5/3x2.5/3X2
EI 22	tissue	sheep	between spleen and liver	Cyst Tissue	2017	Ariha	Male	<1	1x1.5
EI 23	tissue	sheep	between spleen and liver	Cyst Tissue	2017	Ariha	Male	<1	1x1.5
EI 24	tissue	Goat	Liver	Cyst Tissue	2017	Ariha	Male	3	3x3.5
EI 25	tissue	Sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	2x3cm
EI 26	tissue	sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	2x2

Table 3.1.c: Collected cyst tissue samples details:

EI 27	**tissue**	**Sheep**	**Liver**	**Cyst Tissue**	**2017**	**Tulkarem**	**Male**	**<1**	**2x2.5**
EI 28	tissue	sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	1.5x1.5
EI 29	tissue	Sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	2x2
EI 30	tissue	sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	**<1**	**2x2 cm**
EI 31	tissue	Sheep	Omentum	Cyst Tissue	2017	Ariha	Female	<1	2x2
EI 32	tissue	Sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	3.5x3
EI 33	tissue	sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	1.5x1.5
EI 34	tissue	sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	2x2
EI 35	tissue	Sheep	Omentum	Cyst Tissue	2017	Ariha	Female	<1	1x1
EI 36	tissue	sheep	Liver	Cyst Tissue	2017	Tulkarem	Male	<1	3x2
EI 37	tissue	sheep	Liver	Cyst Tissue	2017	Tulkarem	Female	<1	1x1
EI 38	tissue	sheep	Omentum	Cyst Tissue	2017	Ariha	Female	<1	2X2
EI 39	tissue	sheep	Gallbalder	Cyst Tissue	2017	Tulkarem	Male	<1	3X2.5
EI 40	tissue	Sheep	Liver	Cyst Tissue	2015	Ariha	Male	<1	1.5x1.5
EI 41	tissue	Goat	Lung	Cyst Tissue	2015	Ariha	Female	20-Oct	2X2
EI 42	tissue	Goat	Liver	Cyst Tissue	2018	Ariha	Male	<1	3X2.5
EI 43	tissue	Goat	Liver	Cyst Tissue	2018	Ariha	Male	<1	1.5x1.5
EI 44	tissue	Goat	Liver	Cyst Tissue	2018	Ariha	Male	<1	1.5 x 2
EI 45	tissue	Goat	Liver	Cyst Tissue	2018	Ariha	Male	<1	1.5 x 2
EI 46	tissue	sheep	Eosophagus	Cyst Tissue	2017	Tulkarem	Male	<1	1.5 x.1.5
EI 47	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	6	4 x5
EI 48	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	4	1 x 1
EI 49	tissue	Sheep	Liver	Cyst Tissue	2018	Al-Khalil-Yatta	Female	1	5 x 5
EI 50	tissue	Sheep	Liver	Cyst Tissue	2018	Al-Khalil-Yatta	Female	1	1 x 1
EI 51	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	7	4 x 4
EI 52	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	7	4 x 3

Table 3.1.d: Collected cyst tissue samples details:

EI 53	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	9	1 x 1
EI 54	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	7	2 x 2
EI 55	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	5	1 x 1
EI 56	tissue	sheep	Lung	Cyst Tissue	2018	Al-Khalil	Female	1	4.5 x 4.5
EI 57	tissue	sheep	Lung	Cyst Tissue	2018	Al-Khalil	Female	1	1 x 1
EI 58	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	1	6 x 5
EI 59	tissue	Goat	Liver	Cyst Tissue	2018	Al-Khalil-Dura	Female	1	0.5 x 0.5
EI 60	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil-Beit Awwa	Female	1	2 x2
EI 61	tissue	Goat	Liver	Cyst Tissue	2018	Al-Khalil-Areehieh	Female	1	2 x 1
EI 62	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil-Areehieh	Female	3	all 2 x 2
EI 63	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	3	2x2, 2x2, 1x1
EI 64	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	1	1x1
EI 65	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	1	1x1
EI 66	tissue	sheep	Liver	Cyst Tissue	2018	Al-Khalil	Female	2	2x2, 2x2
EI 73	tissue	Sheep	Liver	Cyst Tissue	2018	Ariha	Male	1	2 x2
EI 74	tissue	Sheep	Liver	Cyst Tissue	2018	Ariha	Male	1	2 x 1
EI 75	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	1x1
EI 76	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	1x1
EI 77	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	2x2
EI 78	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	3	1x1
EI 79	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	1x2
EI 80	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	1x2
EI 81	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	1x1
EI 82	tissue	Goat	Gallbalder	Cyst Tissue	2018	Tulkarem	Male	<1	1x2
EI 83	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	2x2
EI 84	tissue	Sheep	Liver	Cyst Tissue	2018	Tulkarem	Male	<1	1x1

Table 3.1.e: Collected cyst tissue samples details:

Eh 96	**tissue**	**Human**	**Liver**	**Cyst Tissue**	**2014**	**Al-Khalil-Dura**	**Female**		

*Tissue samples that are highlighted in yellow perhaps are not be a cyst; they may have been mistakenly extracted. Suspected to be necrotic

3.3 Molecular assay:

3.3.1 DNA extraction

Before performing the DNA extraction, some pre-analysis steps were done (pre-treatment of tissue) due to the complexity of the cyst's double layers which obstruct the extraction of the DNA. After thawing the frozen samples at room temperature, tissue samples were chopped into tiny pieces (0.025 gm - 0.04 gm) for each tissue sample using a sterile surgical blade. The chopped tissues were transferred into a 1.5 ml Eppendorf tube, and 180 µl of lysis buffer T1(NucleoSpin®) was added to facilitate the denaturation of proteins, followed by thermal incubation at 95°C for 10 minutes. A mechanical process followed up, about 0.5 mm of glass beads(Cell disruption media, Scientific Industries, Inc-SI BG05) were added to enhance the extraction and denaturation of proteins, and then a vortex was established using a beater(Vortex-Genie or Digital Distributor Genie) for 5 minutes at the maximum speed 2850 rpm, after shaking, 30 µl of proteinase K was added (22mg/ml), and finally, incubation of the samples in a water bath overnight at 56°C. Then, after the tissue was completely dissolved, samples were picked and stored at -20°C until needed.

After the treatment of tissue samples, they were ready to perform the extraction using the NucleoSpin® tissue kit as described by the manufacturer(*NucleoSpin Tissue Kit*t®). After the extraction, DNA samples were stored at -20°C until needed (see Appendix 1).

3.3.2 Screening PCR

The genomic DNA of *E. granulosus* from cyst tissue samples was amplified according to(Abbasi et al., 2003), by using universal primers Eg2691 upstream 5′- ACACCACGCATGAGGATTAC-3′ and Eg2692 downstream 5′-ACCGAGCATTTGAAATGTTGC-3′, targeting the EgG1 *Hae* III sequence, 266 bp to confirm the presence of the *echinococcus* parasite molecularly within collected samples, these primers are widely used because of their stability(Mobedi et al., 2013),(Jenkins et al., 2014). Samples were prepared for amplification by mixing 12.5 µL of Taq PCR red mix (PCRBIOSYSTEMS®), 1.5 µL of mix primers (15uM) that were previously prepared, 8.5 µL of de-ionized water, and 2.5 µL of extracted DNA for each sample, the experiment included positive control (mixture with known DNA) and negative control (mixture without DNA) samples. The amplification process was performed in three stages: the first stage is the complete denaturation of the mixture at 95°C for 5 minutes in one cycle. The second stage consists of 35 cycles as followed: 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C, and the third stage is final elongation for 10 minutes at 72°C in one cycle, samples incubated at 4°C. 5 µL of PCR products were applied to 2% agarose gel (2 gm agarose powder dissolved in

100 ml of diluted 1 x TBE buffer) with 4 drops of ethidium bromide (10mg/ml). Five (5µL) of 100 bp ladder(PCRBIO®) were used as DNA size markers and then were visualized by UV light(UVITEC Cambridge from Alliance®), (see results, **Figure 4.1 (a), (b)**).

3.3.3 Sanger sequencing

Random positive samples resulting from the screening PCR were selected to undergo Sanger sequencing to ensure the specificity of our work. Data obtained from the Sanger sequencing were received as FASTA files, which were then uploaded into NCBI-BLAST, a basic local alignment search tool(Search Nucleotide Databases Using a Nucleotide Query, n.d.) to confirm the homology between our sample sequence results (PCR product) with known reference sequences of the *echinococcus* genome published by GenBank (see results, **Figure 4.2**).

3.3.4 Sample preparation for NGS:

NGS was the method of choice, due to its features in sequencing each amplified fragment with high-quality throughput to detect polymorphic regions of the target region in one round. The background of the research is represented in two different PCR systems that were performed by a generation of amplicon multiplexing PCR, referring to the globally used mitochondrial genes of the parasite NADH dehydrogenase subunit-1 (NAD-1) and cytochrome c oxidase subunit-1 (Cox-1) in targeting and amplifying the short targeted fragments regions (SNPs) and later for analysis and differentiation between genotypes through efficient sequencing method by the next-generation sequencing (NGS), which followed by bioinformatic analysis.

The NGS system's role in amplification is to amplify short fragments of the targeted genome as clusters, so the first step was preparing the desired amplicons from targeted DNA samples according to the size of the NGS platform kit used (Illumina®) usually between 120 bp to 150 bp in length, for this purpose, special primers sequences were successfully designed to amplify the targeted regions of the genome, and amplified by polymerase chain reaction (PCR), the primers designed based on mtDNA of the parasite COX-1 and NAD-1 that were used for several years to investigate the genetic variants of different species of the parasite.

3.3.4.1. NGS-PCR primer design & amplification:

Multiple sequences published in NCBI GenBank of *E. granulosus* species were obtained to create a stable, producible system in detection of the informative polymorphic regions using the specific Single nucleotide polymorphisms (SNPs) position and confirming the specificity of genotyping, sequences were then aligned to detect the SNPs presence for each gene using MultAlin software, (see results, **figure 4.3**). The published GenBank sequences for each gene COX-1 and NAD-1 were applied to MultAline software to define informative polymorphic regions for each, ending with marking all desired SNPs on each gene sequence to establish a system that we can depend on to extract the SNPs in our isolates, if they exist or not. Referring to that, we could differentiate between our samples adopting which parasite's genotype.

After detecting each gene's informative polymorphic regions, specific primers were designed using Prime-3 software (website), to choose the most appropriate primers according to the data inserted in the desired position. A sum of 10 primers grouped as five PCR segments of COX-1 and NAD-1 genes were designed to generate amplicons for multiplex PCR to amplify the desired segments containing the informative SNPs used as markers for specific genotypes. All primers were modified with overhanging Illumina adaptor sequences at the 5' ends of the forward (5'-CGTCGGCAGCGTCAGATGTGTATAAGAGACA-3') and reverse primers (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-3'). The first designed primers had to cover SNPs which are located in the first fragment because of the desired fragments' length of the used NGS platform (120-150 bp), for example, PCR amplicon 1 from **Table 4.1** (NAD1_SNP1_2NGSF, and NAD1_SNP1_2NGSR) forward and reverse primers covered the first two SNPs in NAD-1 gene polymorphic region, and so on for each designed primer. Three PCR amplicons were generated from the NAD-1 gene, and two are from the COX-1 gene (see **Table 4.1**). Two pools were generated, the first Pool mix (Pool mix-1) contains NAD1-region1 and NAD1-region3 with COX1-region1, and the second Pool mix (Pool mix-2) consists of NAD1-region2 mixed with COX1-region2 primers. The purpose of separating the primers into two pools is to prevent the primers from overlapping. (see results, **Figures 4.4 (K) and (L)**).

3.3.4.2. The first PCR:

The first amplification process (1st PCR) was performed through the preparation of two groups, the first included the adding of 1.5 µL of DNA to a mixture of 12.5 µL of PCR red mix, 8 µL of de-ionized water, and 3 µL (30uM) of Pool mix-1 in a 0.2 ml PCR tubes. The second group was performed by the addition of 1.5 µL of the same DNA samples into a 0.2 ml PCR tube with 12.5 µL PCR red mix, 8 µL of de-ionized water, and 3 µL (30uM) of Pool mix-2. PCR conditions of this stage were: 95°C for 2 minutes for the first denaturation cycle. The middle stage was performed in 35 cycles at 95°C for 15 seconds, 56°C for 15 seconds, and 72°C for 45 seconds. The last stage is the final elongation in one cycle at 72°C for 5 minutes. Gel electrophoresis was followed to visualize the desired bands under UV light using a 2% agarose gel stained with ethidium bromide (see results, **figure 4.5**).

3.3.4.3. Magnetic beads first purification:

The two amplified groups were combined and purified using magnetic beads and eluted in 25 µL of elution buffer. The end clean product will be used as a template for the second PCR to add specific DNA sequence barcodes for each sample. These barcodes will be used to demultiplex the samples and separate them in Fastaq formats. Cleaning steps carried out as follows: 20 µL of each pooled sample was added into a new 0.2 ml PCR tube with 50 µL of 1X AMPure XP beads (AMPure XP Beads®), 30 µL of de-ionized water was added and left at room temperature for 10 minutes. Sample tubes were then put onto a magnetic plate (DNA attached to beads which attached to the magnetic plate). After 2 minutes, the clean supernatant was discharged. Followed with the addition of 180 µL of freshly prepared 80% ethanol was applied twice for washing the samples while the tubes on the magnetic plate were left to dry at room temperature for 5 minutes. After

that, 25 µL of Elution buffer was added after transferring the samples to a regular rack and waiting for 5 minutes at room temperature on the bench. Samples were placed back on the magnetic plate again to take the supernatant which carried the DNA into a new 0.2 ml PCR tube that will be labeled with indices to perform the second PCR.

3.3.4.4. Second PCR:

After cleaning, 7.5 µL out of 25 µL of cleaned products were placed into a new 0.2 ml PCR tube mixed with 12.5 µL of PCR red mix, and 2.5 µL of two different indexes for each sample. A second PCR was then performed in four stages as follows: the complete denaturation of the mixture in the first cycle was performed at 72°C for 3 minutes, the second stage was at 95°C for 30 seconds in one cycle, the third stage in 10 cycles, at 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, the last stage was one cycle at 72°C for 5 minutes.

Table 3.2 Samples encoded with two different indexes for each as shown below:

Indexes	711	712	714	715	716	718	719	720
502	1	2	3	4	5	7	8	9
503	10	11	12	13	14	15	16	17
505	18	19	20	21	22	23	24	25
506	26	27	28	29	30	31	32	33
507	34	35	36	37	38	39	47	48
508	49	50	51	52	53	54	55	56
Indexes	701	702	703	704	705	706	----	----
521	40	42	43	44	45	46	----	----

3.3.4.5. Magnetic beads second purification:

Pooling and cleaning of PCR products that have indexes were performed by taking 5 µL of each sample into a 1.5 ml Eppendorf tube and mixing them in the same tube with beads at a 1:1 ratio. Cleaning step-2 was then performed as mentioned before in the same way as cleaning step-1, ending with eluting the DNA by adding 25 µL elution buffer. Pure, barcoded, cleaned DNA was collected and sent for deep sequencing for 150bp length by NGS to an outside company, like Macrogen©, on Illumina machine.

3.3.5 Bioinformatic analysis and genotyping

Sequenced data by NGS was received as FASTQ files, which present the quality of readings for each nucleotide per sequence. Data Analysis was processed by the bioinformatics software Galaxy (https://usegalaxy.eu/) through a designed workflow. Millions of reads were analyzed as a dataset collection to apply the workflow on all samples at once. Sequencing and data analysis were done

based on the single-nucleotide polymorphisms (SNPs) variants result of each sample, to build a database of genetic variation which facilitates understanding the prevalence of the predominant genotype of *E. granulosus* species and genotypes in the IMH in the West Bank of Palestine. After receiving FASTQ files from sequencing by NGS, the files were processed using a pre-designed workflow that analyzed the data using Galaxy software. The output files (FASTA files) were mapped by running the inserted workflow based on the gene references needed. According to the traditional analysis method, data will be visualized by IGV software (IGV_2.17.4), consensus sequences of each sequence were copied to MultAlin (website) to reveal the genotype and SNPs positions by alignment of these sequences according to references genes (COX-1, NAD-1), genotype then determined according to variants appearance and counts per sample (see **Appendix-2**) of conventional way of genotyping analysis. But not anymore, the whole analysis process was done with one click, through a designed workflow plan on Galaxy (see **figure 3.1**). the first step was to upload your received files from NGS as FASTQ files on Galaxy and collect them in one file called a dataset collection, then our data will be mapped to reference sequences to enable comparing the differences between the wild type reference (G1 genotype in our case) with our sequences. The base call variants will recognize and gather all the variants or mutations and it's format on each sequence against the selected reference gene to finally select the desired base format (as inserted in workflow programming) and show them as a data file result. The last step was to select what result you want to display, we ordered the workflow to present only the "not-matching" bases to select our variants within several variants in the final results files. The analyzed data was subjected to statistical analysis to evaluate the resulting values and ensure high-quality results. In just a few hours, you can reveal the sequenced sample's genotype, quality assessment, and statistics (see genotyping results **figure 4.9**).

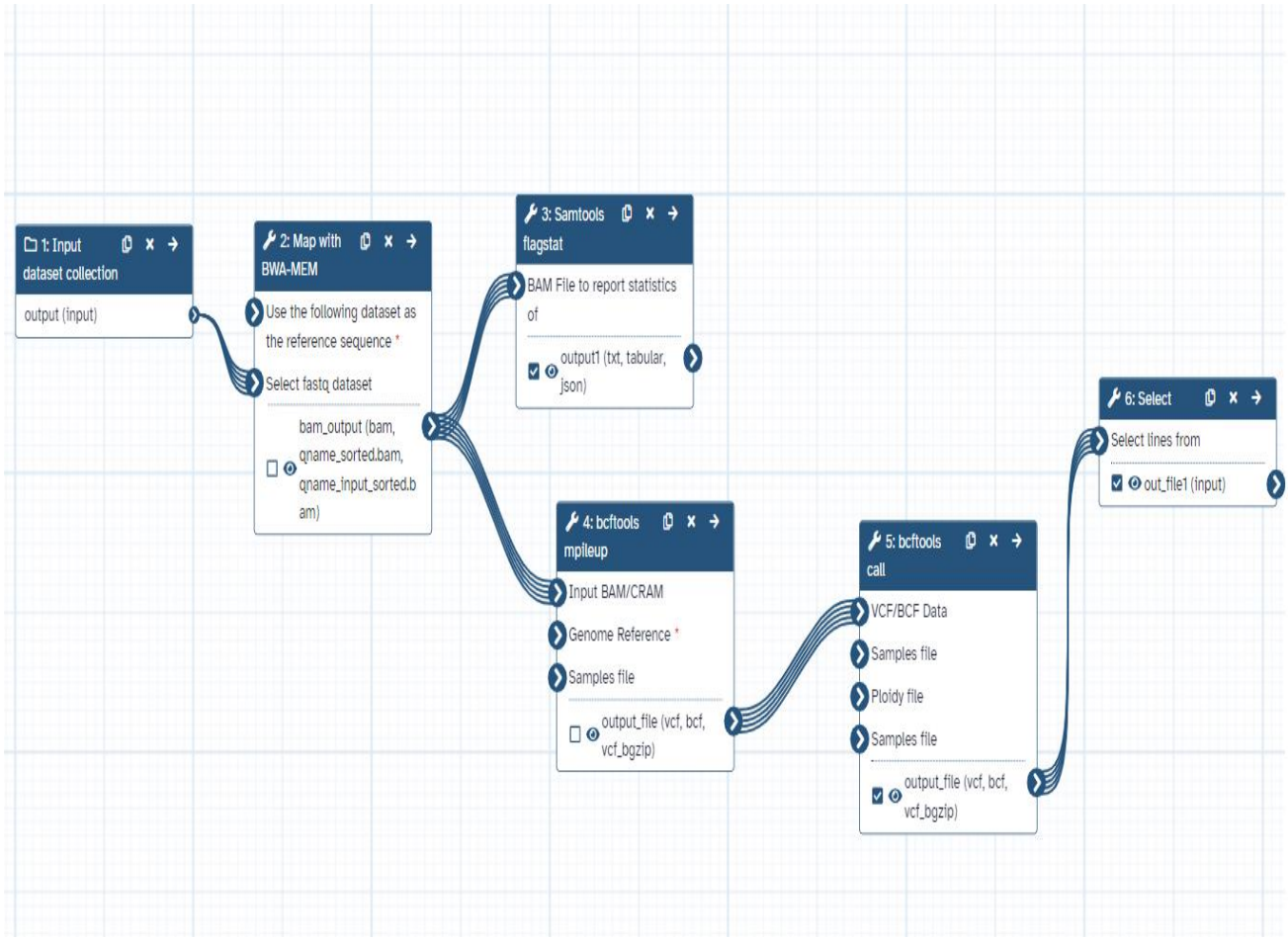


Figure 3.1 The workflow map scheme for FASTQ files analysis on Galaxy software.

Chapter Four:

Results

Many explanations refer to the near relationship between species, represented in a low degree of polymorphism in the mitochondrial genome. However, the amplicon directed at the Cox-1 gene differed from its corresponding G1 sequence by two nucleotides (C > T, T > C) at positions 77 and 268 bp from the sequence beginning respectively, whereas the Nad-1 amplicons differed by five nucleotides when compared to its corresponding G1 sequence (G > C, A > G, G > A, A > G, G > A) at positions 32, 55, 397, 645 and 654 respectively (see **figure 4.3 (I) and (J)**). Therefore, a multiplex PCR system was generated to avoid cross-activity with tissue samples. **Figure 4.4** shows two PCR systems of **(K)** NAD1-2 with COX1-2 (Pool mix-2) and **(L)** NAD1-1 and NAD1-3 with COX1-1 (Pool mix-1) to detect NAD1-1 fragment SNPs.

4.1 Screening PCR

Forty-seven samples were successfully amplified targeting the *Echinococcus* sequence EgG1 Hae III, 266 bp, according to(Abbasi et al., 2003). Screening the genomic DNA of *E. granulosus* within tissues visualized through a 2% agarose gel under UV light.

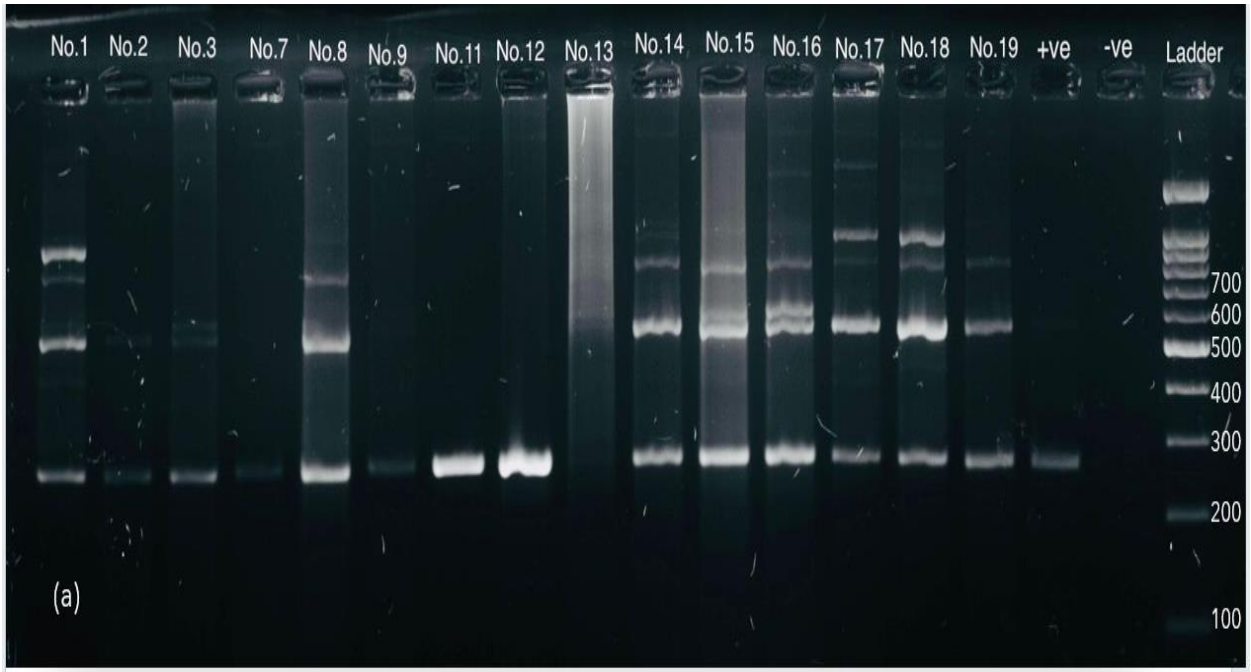


Figure 4.1 (a), (b): Gel electrophoresis of screening PCR represented positive sample results after amplifying the EgG1 *Hae III* sequence, 266 bp by PCR.

4.2 Sangar sequencing:

After Sanger sequencing of random positive screening samples was done, the National Library of Medicine (NIH) nucleotide blast tool was used to confirm the attendance of the parasite molecularly over the obvious matching results between our Sanger-sequenced samples and published sequences of *e. granulosus* species by the National Center for Biotechnology

Information (NCBI) at high percentage as follows(Nucleotide BLAST, NCBI).

C

Echinococcus granulosus isolate Q8 EgG1 HaeIII repeat region	Echinococcus granulosus	315	526	30%	1e-80	98.31%	178	KX017218.1
Echinococcus granulosus isolate K9 EgG1 HaeIII repeat region	Echinococcus granulosus	311	522	30%	2e-79	97.75%	178	KX017220.1
Echinococcus granulosus repeat region	Echinococcus granulosus	243	243	13%	6e-59	97.86%	140	DQ009619.1

D

Download GenBank Graphics Sort by: E value

Echinococcus granulosus isolate Q8 EgG1 HaeIII repeat region

Sequence ID: KX017218.1 Length: 178 Number of Matches: 2

Range 1: 1 to 178 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
315 bits(170)	1e-80	175/178(98%)	0/178(0%)	Plus/Plus

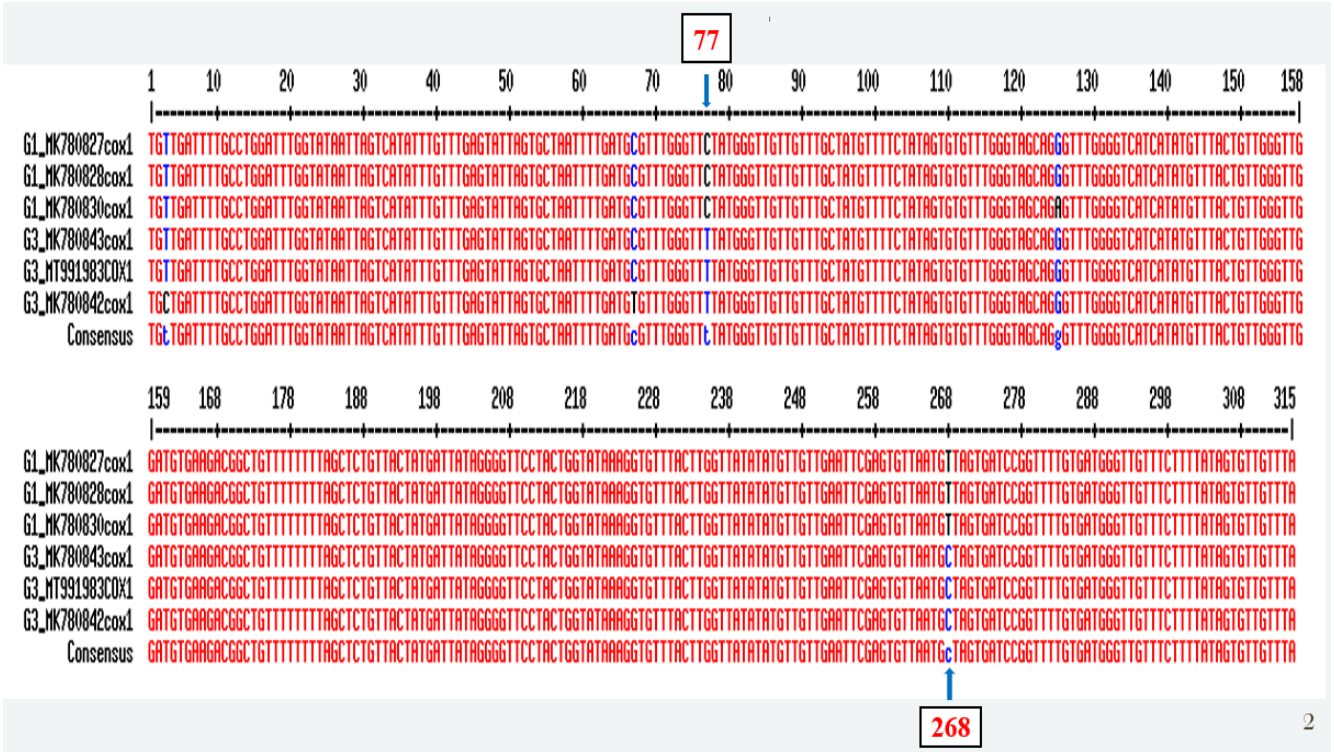
```
Query  273  CATTTAACTTACCACTGGGCCGTGCTGTGGAGGTAATTTCGGCATCTAGTGAAGGAAAAA  332
            ||||||||||||| |||||||||||||||||| ||||||||||||||||||||||||
Sbjct  1    CATTTAACTTACCNGTGGGCCGTGCTGTGGAGGTAGTTTCGGCATCTAGTGAAGGAAAAA  60

Query  333  TAAGACATCGGTGCGAGCACTCCTTCTCACTCATCTCACTGAATGTGAAATATCTCCACC  392
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TAAGACATCGGTGCGAGCACTCCTTCTCACTCATCTCACTGAATGTGAAATATCTCCACC  120

Query  393  TGACGAAGGCAACTTCACTGCCATTGCCCCCAAGATGCAACATTTCAAATGCTCGGTA  450
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TGACGAAGGCAACTTCACTGCCATTGCCCCCAAGATGCAACATTTCAAATGCTCGGTA  178
```

Figure 4.2 Sangar sequencing of positive screening samples **[C, D],** high homology of cyst tissue sample estimated at 98% compared with *e. granulosus* isolate EgG1 *Hae III* repeat region sequence (Acc. No. KX017218.1). Another sample was sequenced and showed a homology of 100% with *e. granulosus* repeat region sequence (Acc. No. DQ157697.1), sequence length 269 bp. The final sequenced sample showed a homology of 97.59% with *e. granulosus* another isolate repeat region sequence (Acc. No. KX017220.1).

4.3 Primer design

Selective primers were designed after locating the position of informative polymorphic regions according to **3.3.4.1** in methodology, SNPs (**Figure 4.3 (I), (J)**). Designed primer's sequences are shown in **Table 4.1** linked with adaptors for NGS. The primer's location is declared on the gene sequence (see **Figure 4.4**).

(I)



(J)

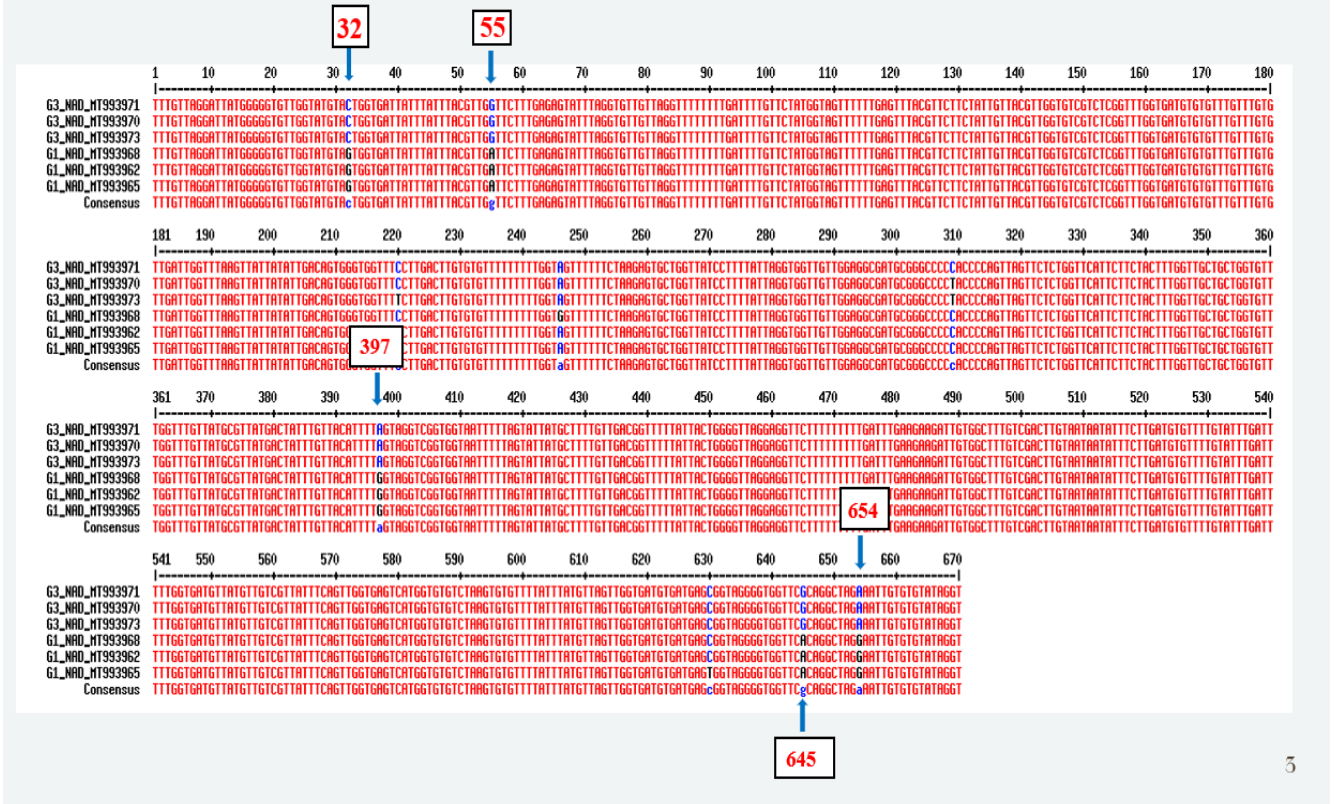


Figure 4.3 (I) Highlighted informative SNPs 77 and 268 of *E. granulosus* mtDNA COX-1 gene

using GenBank virtual sequences through MultAlin software, and **(J)** Highlighted informative SNPs of NAD-1 32, 55, 397, 645, and 654, using GenBank virtual sequences through MultAlin software.

Table 4.1: The designed primers are linked with adaptors, highlighted sequences in yellow are for the designated primers, and the adaptors' sequences are the rest. F: forward; R: reverse.

PCR amplicons	Primer sequences	Amplicon size (bp)	Targeted gene
RCR amplicon 1	>NAD1_SNP1_2NGSF TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGGGCGGTGATTATGTTGG GTT >NAD1_SNP1_2NGSR GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGCAAACCGAGACGACA CCAAC	205+76 bp	Mitochondrial gene NAD-1
PCR amplicon 2	>NAD1_SNP3NGSF TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGTTGCTGCTGGTGTTTGGT TT >NAD1_SNP3NGSR GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGACAAGTCGACAAAGCC ACAA	161+76 bp	Mitochondrial gene NAD-1
PCR amplicon 3	>NAD1_SNP4_5NGSF TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGCAGTTGGTGAGTCATGG TGTG >NAD1_SNP4_5NGSR GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGACAAACCAAGAACCA CAGCA	190+76 bp	Mitochondrial gene NAD-1
PCR amplicon 4	>Cox1_SNP1NGSF TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGTGTTGATTTTGCCTGGAT TTGG >Cox1_SNP1NGSR GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGCAGCCGTCTTCACATC CAAC	174+76 bp	Mitochondrial gene COX-1
PCR amplicon 5	>Cox1_SNP2NGSF TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGGTTGGATGTGAAGACGG CTG >Cox1_SNP2NGSR GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGATGAGCCACCACAAAC CAAG	244+76 bp	Mitochondrial gene COX-1

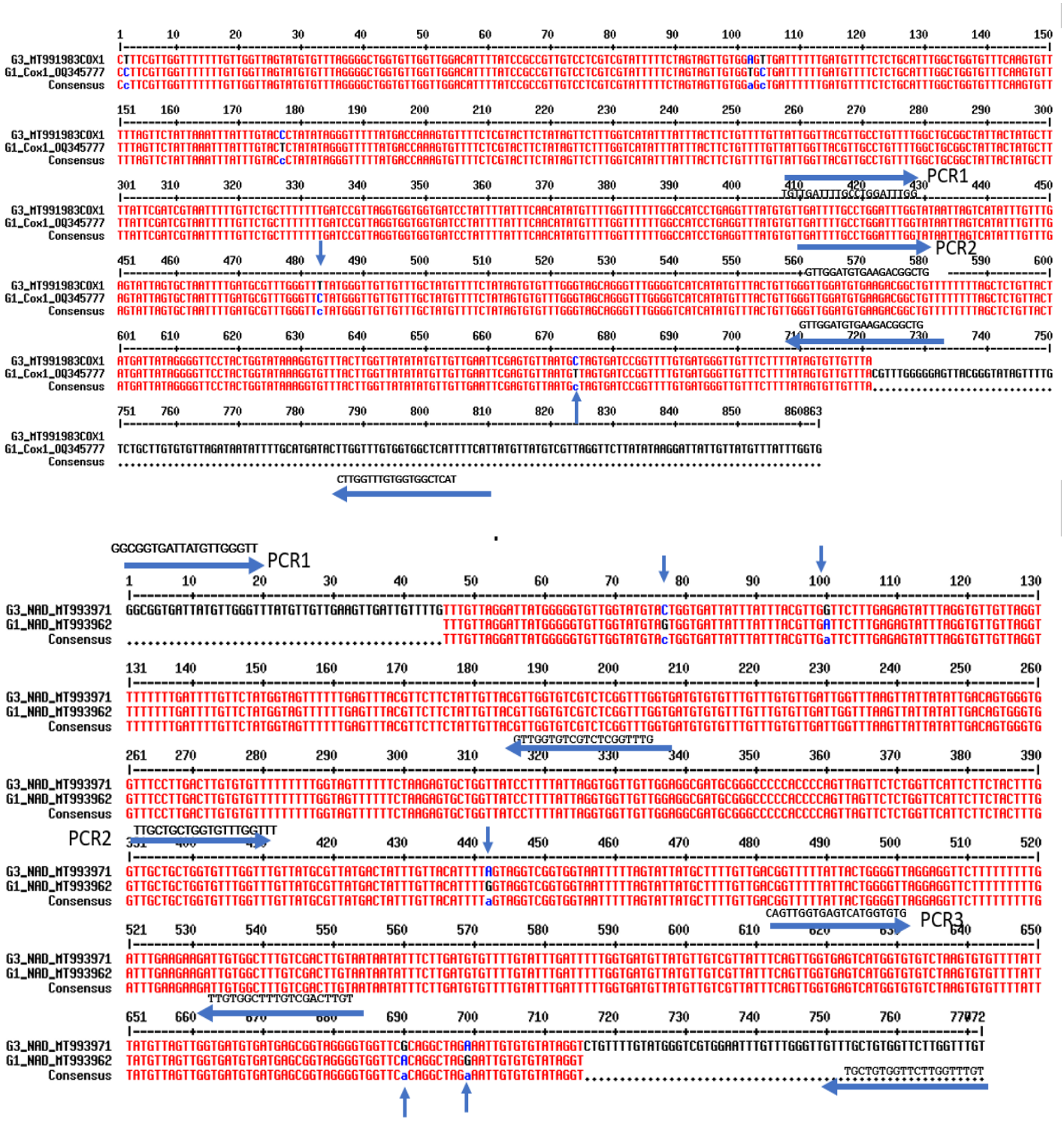


Figure 4.4 Designed primers' location on both genes' sequences represented in two PCR systems within five amplicons. Horizontal arrows in blue present the sequence of the designed primers that will amplify specific regions of the mitochondrial genes **(K)** COX-1 and **(L)** NAD-1, Forward and Reverse.

4.4 Gel electrophoresis results of the first PCR

The first amplification process carried out using pool mixtures of the designed primers of each gene (COX-1, NAD-1), produced fragments of the expected sizes [205+76, 161+76, 190+76] bp for pool mix-1 and [174+76, 244+76] bp for pool mix-2 (see **Figure 4.5**). The desired band was approximated; it may refer to primer dimer or gel sensitivity.

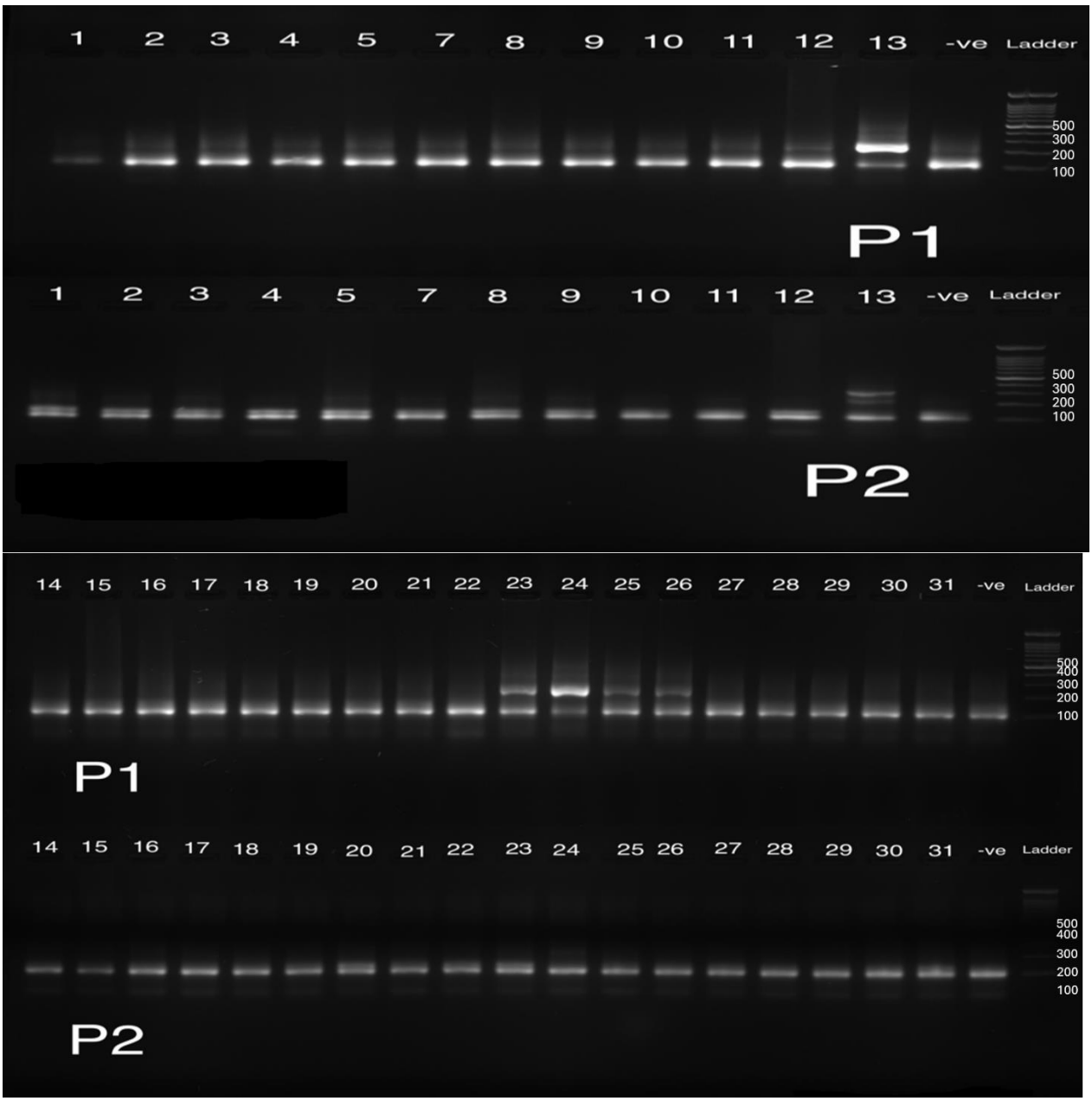


Figure 4.5 Multiple bands produced by different PCR amplicons with expected sizes of (205+76, 161+76, 190+76) bp and (174+76, 244+76) bp for NAD-1 and COX-1 amplicons respectively, P is for pooling.

The main purpose of pooling primers in two separated mixtures is to dissociate similar gene regions from each other to avoid the primers' overlapping.

4.5 Genotyping

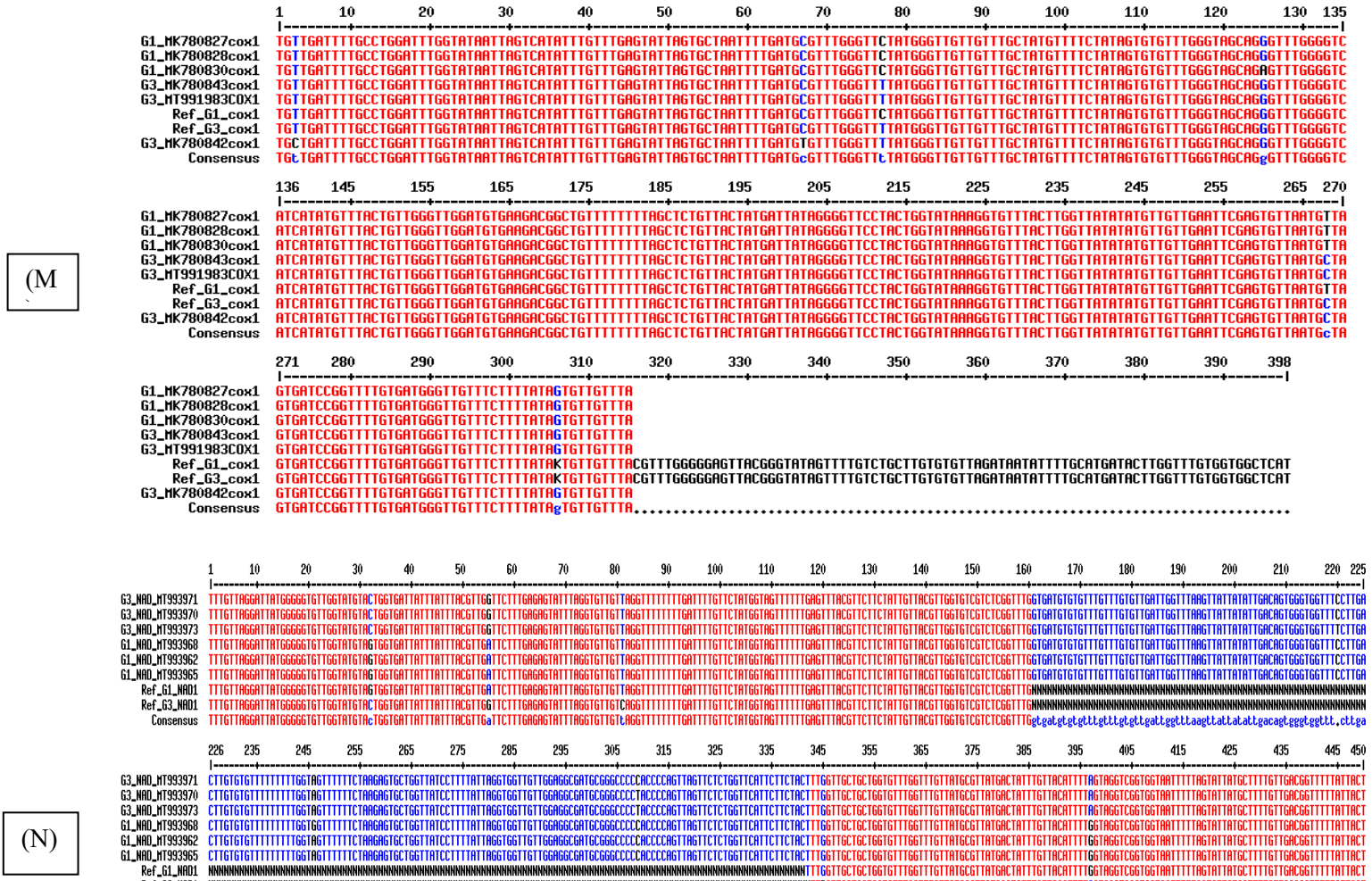


Figure 4.6 (M) Defined SNPs positions of COX1 gene (SNP-77, SNP-268) represent the matching of reference real DNA sequences genotype **G1** and **G3**, nucleotide arrangement at SNPs positions (C>T), (T>C) respectively, referring to the virtual sequences and **(N)** represent the matching between SNPs positions of NAD1 gene (SNP-32, 55, 397, 645, 654) of reference sequences genotype **G1** and **G3** and virtual sequences, nucleotide arrangement at SNPs positions (G>C/

A>G/ G>A/ A>G/ G>A) respectively.

The international DNA samples (G1, G3, G4, G5, G6/7, and G10) were also analyzed by the same system method pathway, they were used as a standard to assess the method's efficacy and sensitivity (see **figures 4.7 and 4.8**).

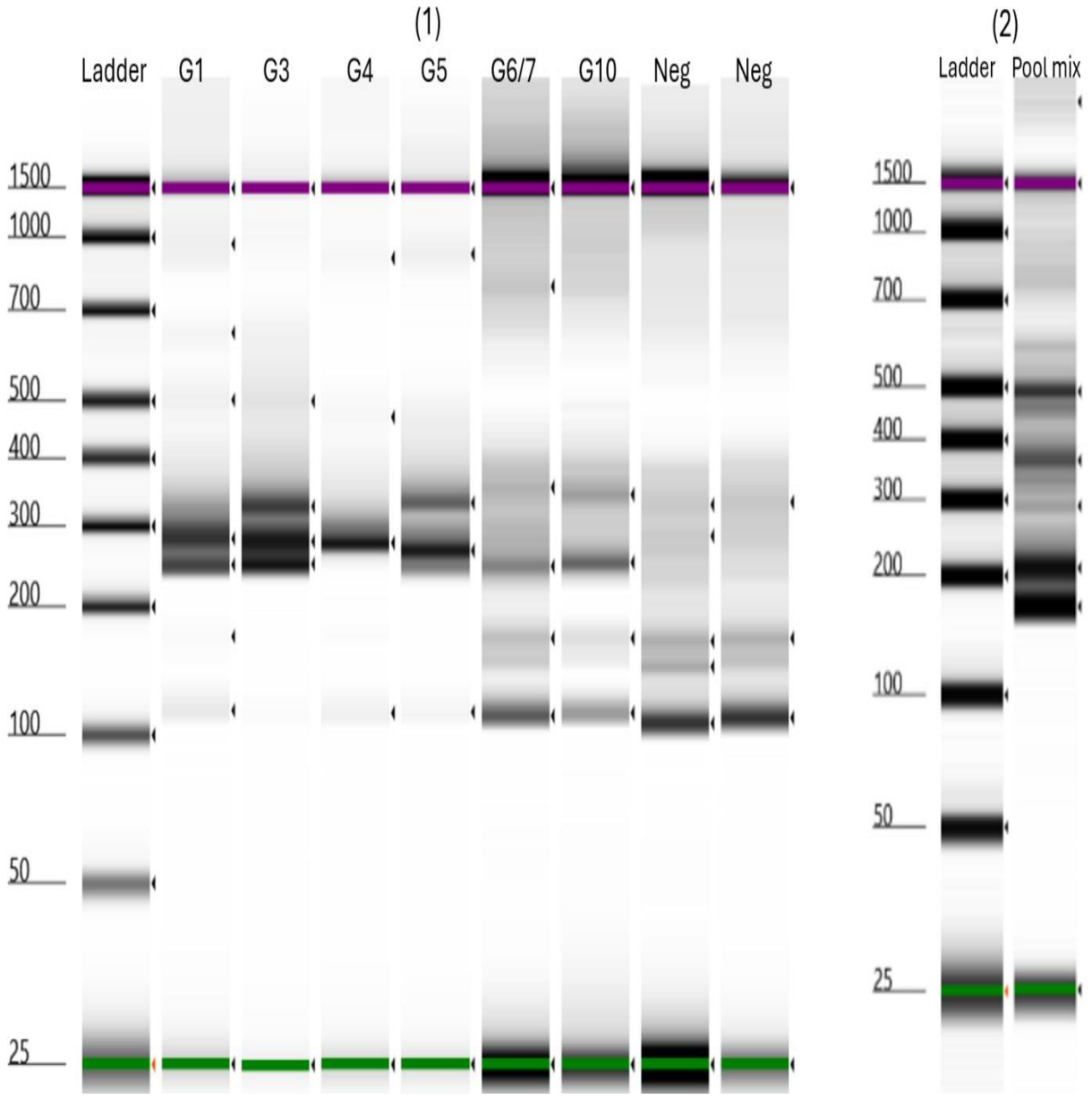


Figure 4.7 NovaSeq Pool Library Quality Control Analysis by Tapestation electrophoresis, **(1)** first PCR of WHO-DNA donated samples, showing the approximate desired band in the range size. **(2)** all DNA samples pooled together in one Eppendorf tube after the second PCR (adding indexes).

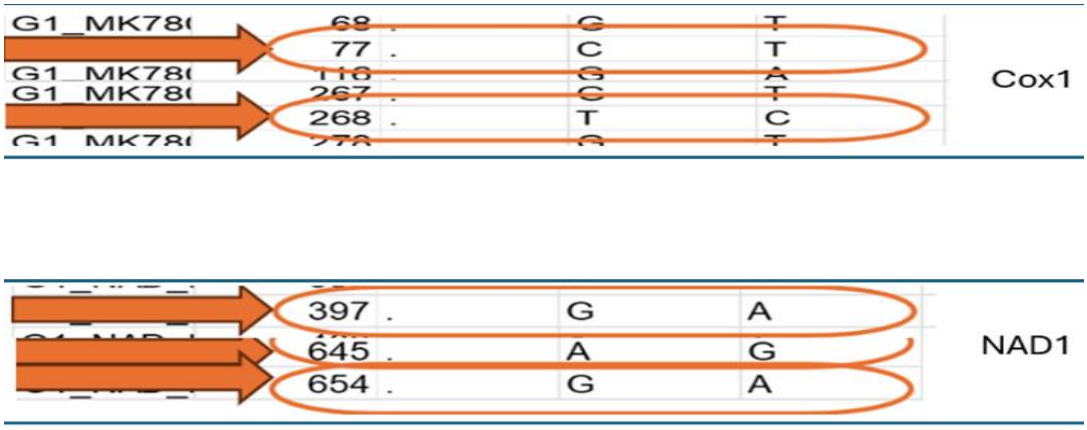


Figure 4.8 Genotyping analysis software results by Galaxy workflow of the international WHO-DNA donated samples, SNPs highlighted in orange. (I) analysis of G10 genotype samples showing both SNPs of COX-1 gene (77: C > T, 268: T > C), (II) G10 genotype analysis according to NAD-1 gene SNPs found (397: G > A, 645: A > G, 654: G > A).

The following figure is an example of one sample genotyping analysis that went through a software analysis by Galaxy workflow after NGS sequencing representing the exact position of the previously defined informative variants for both genes COX-1 and NAD-1, this resulted in the conclusion of that, this sample is G3 genotype and no conflicting results of the same samples for two genes, and thus the same to the rest of the samples with a principle of at least four SNPs out of seven.

#CHROM	POS	ID	REF	ALT
G1_MK78(	67	.	C	T
G1_MK78(	77	.	C	T
G1_MK78(	268	.	T	C

#CHROM	POS	ID	REF	ALT
G1_NAD_I	32	.	G	C
G1_NAD_I	55	.	A	G
G1_NAD_I	397	.	G	A
G1_NAD_I	633	.	T	C
G1_NAD_I	645	.	A	G
G1_NAD_I	654	.	G	A

Figure 4.9 Genotyping analysis format result of one tissue sample COX-1 SNPs in the right positions {77: C > T, 268: T > C} highlighted in green and {32: G > C, 55: A > G, 397: G > A, 645: A > G and 654: G > A} for NAD-1 SNPs positions, highlighted in red and orange. POS stands for position, REF is for reference gene sequence (G1 in that system), ALT is for alternative nucleotide (the variant), and QUAL is for quality assessment of reads.

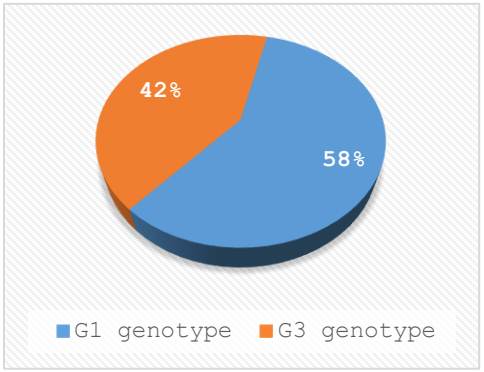


Figure 4.10 A pie chart of the genotyping results showing a high incidence of G1 genotype, about 58%, and G3 forming 42% of sequenced samples.

Chapter Five:

Discussion

Genetic and epidemiological studies are a must since the disease is still prevalent; for this purpose, a novel method was invented to identify the polymorphic regions among the parasite species to facilitate diagnosis and treatment evaluation. The new technology has ensured its effectiveness with high sensitivity and specificity which could significantly improve the ability to establish efficient diagnostic and treatment management methods that in turn may actively help in restricting the pathogen's prevalence in pastoral areas. Evolution could bring its goodness to all who are concerned, such as people in laboratory settings and the health sector, facilitating paths for epidemiological studies.

Discrimination of *e. granulosus* species by aiming at the genetic variation of the parasitic mitochondrial genome through bioinformatic analysis could present a process of precise identification and genotyping, see results of genetic variation of the COX-1 and NAD-1 genes, which revealed seven different SNPs in targeting and detection of E. granulosus s.s species G1 and G3 genotypes. Selected designed primers amplified the desired size of amplicons, followed by NGS sequencing (see detailed primers with their adaptors, **Table 4.1**)

Our observations on resulting variations are in accord with phylogenetic studies on the genome level after sequencing, which elucidated a high incidence of *E. granulosus s.s* genotypes **G1** and **G3** in the West Bank of Palestine among intermediate and aberrant hosts (see **figure 4.10**). This developed system format enabled prosperous and efficient diagnosis for *Echinococcus* species in a single tube, a parallel step resulting in highly sensitive and specific outputs that improve reporting of infected cases on the genotype level, advance our knowledge on the epidemiology of these parasites, and ultimately support effective control of CE.

According to screening PCR, 98% of samples were successfully amplified, showing positive results of the expected band size. Multiple bands shown may refer to the nature of the *Echinococcus* genome's tandem repeat by 133bp -Al-Abbasi *Hae* III band-(Abbasi et al., 2003),

as shown below (see **figure 4.1 (a), (b)**).

After applying Sanger sequencing to our randomly selected positive screened sequences, we have proved molecularly that our bands on gel electrophoresis are not artifacts; they belong to the *E. granulosus* species, meaning that the cyst was formed by the *Echinococcus* species. The resulting fragment length of 269 bp, shown in **(Appendix 3, F),** was amplified using(Abbasi et al., 2003). The screening primers were eg269; this length match indicates the specificity of the results that refer to *E granulosus* sequence, sequence alignment reviewed matches with *E. granulosus* short fragments from Gene Bank.

Table 4.1 summarizes the exact sequence of each resulting designed primer with the expected size of the resulting amplicons, including the adapters that were added to facilitate the attachment of inserted samples into the flow cell of the NGS device. The adaptors are complementary to oligos on the flow cell to perform the effective sequencing needed. The position of primers on the gene sequences is shown in **Figure 4.4 (K)** and **(L),** which clarifies every informative SNP firmed position distance away from the beginning of amplification where the primer is localized.

Before performing data analysis and genotyping, ensuring the system integrity was done by a homology comparison between real sequences of DNA with Gene Bank virtual sequences, which confirmed the effectiveness and stability of our workflow system, using MultAlin website (see **Figure 4.6 (M)** and **(N)**) that affirmed the homology between the real DNA reference sequences and the virtual ones at SNPs positions. International DNA samples of *E. granulosus* genotypes which were kindly donated by The Epidemiology Collaborating Centre-Detection, and Control of Cystic and Alveolar Echinococcosis (in humans and animals) of the World Health Organization, Higher Institute of Health, Rome, Italy, as reference sequences of *E. granulosus* genes (**G1**, **G3**, **G4**, **G5**, **G6/7**, and **G10**), they also went through the same analysis steps by NGS and workflow to be used as a standard to assess the efficiency and specificity of the workflow system before applied on the intermediate host's sequenced samples (**figure 4.8**). Automated electrophoresis was done for all six DNA samples and showed amplified results (**Figure 4.7 (1) and (2)**). Genotyping analysis results recorded the appearance of many of the desired polymorphic regions, which reflects the power of NGS methodology in massive data sequencing in parallel within a short period of time, as so on for genotyping analysis. For the international DNA samples, NGS could detect many SNPs of each, and matching was found with our findings, it reflects the power of NGS in detecting new SNPs of several genotypes and species, which facilitates constructing a whole system of healthcare programs. It's worth mentioning a substantial feature of NGS is the ability to detect several genotypes in the same sample, producing separate files' results that will be very beneficial in the perception and detection of co-infection.

Tapestation electrophoresis uses micro-capillary tubes which introduce evident results, **(1)** the first diagram showed clear, manifest bands of pooled primers, two PCR systems (according to 3.3.4.1 section, first PCR) of samples (G1, G3, G4, G5, G6/7, G10 and the last are two negative control samples, after ladder). (2) The second diagram represents a pool mix of all samples with indexes after the second PCR and cleaning to prepare samples for sequencing (according to **3.3.4.4,** the second PCR section).

WHO-DNA samples genotyping was identified according to the previously mentioned SNPs in **Figure 4.3** by the same analysis pathway, NGS sequencing followed by software analysis. G1 and

G3 were positively confirmed and the other genotypes (G4, G5, G6/7) showed some of the defined SNPs and more new SNPs, normally due to differences between the parasite genotypes' sequences. However, the G10 genotype had five SNPs of interest (**Figure 4.8**), this matching between the variety of *e. granulosus* species genotypes' SNPs and the international WHO-DNA samples emphasized the sensitivity and stability of NGS. Negative samples indeed had no results, which reflects the specificity and accuracy. These results furthered another proof of the system's competence and high performance.

After all, NGS has revealed the presence of *E. granulosus s.s* species in Palestine, 84.9% of sequenced samples were successfully genotyped, and each of them was sorted as a type of *E. granulosus s.s* genotype, **G3** genotype found to be at a relatively high incidence rate (42%) for the first time in Palestine. However, the **G1** genotype still presides over the highest rate prevalence (58%) of samples and predominates in pastoral regions. **Figure 4.9** represents the specificity of the methodology used for genotyping which can pinpoint the exact SNPs in their defined positions to determine the widespread genotype of a specific category in one click, which will save time and effort needed for many hours of analysis.

Noteworthy, all SNPs from two different mitochondrial genes confirm one type of genotype for each sample, and none of the sequenced samples showed conflict in results between COX-1 and NAD-1 in variation analysis, reflecting the system's specificity and efficiency for a specific genome's targets, as shown in **Figure 4.9** as an example of our results.

A recent study in 2020 confirmed the stability of the informative SNPs' position using the mitochondrial nad5 gene, amplified 28 hydatid animal and human cyst samples, and genotyped by different genotyping approaches using Sanger sequencing. 16 of the sequenced samples were identified as G1 genotype, and 12 were G3 genotype(Bonelli, Giudici, et al., 2021). The identical sites of the mitochondrial gene NAD subunits, compared to our findings, reflect a high stability of the informative regions of the same gene, which is an additional feature of the powerful method used in the paper.

Chapter Six:

Conclusion and Recommendations

In conclusion, the study sought to address a knowledge gap of a zoonotic pandemic disease that took millions of lives around the world for many centuries, molecular quantitative assessments of sequencing and genotyping of the parasite's species cause cystic hydatidosis to the intermediate host were used to study the parasite prevalence in the West Bank of Palestine. The study showed that 84% of the cysts belong to Echinococcus parasites as a causative agent in sheep. Previous research showed a worldwide prevalence of CE, especially *E. granulosus s.s* which recorded the largest number of human cases and is still ascending. Surprisingly, the **G3** genotype showed a high prevalence recently in Palestine at 42% compared with previous studies, which introduced 14% of the G3 genotype in 2013(Adwan et al., 2013), Al-Jawabreh et. al succeeded in revealing the **G3** genotype in only two human cyst tissue samples out of 13 collected from different hospitals in Palestine in 2015 using the mitochondrial cytochrome c oxidase subunit 1(Al-Jawabreh et al., 2017). However, **G1** genotype still leads the international genotype distribution trend, as submitted in this study (**Figure 4.10)**.

Molecular studies and discoveries facilitate the establishment of control and awareness programs based on a clear understanding of the disease, which assists in the proper intervention. This study introduces a significant new method resulting in sensitive and specific outcomes that can analyze the majority of samples and its specificity in discrimination of all genotypes found in a specific region with only two steps PCR method which has been proven using global DNA samples, and later by applying to local samples with high throughput in a noticeably short period, using the NGS sequencing, which followed by software analysis for genotyping purposes. The *echinococcus* mitochondrial genes have been used for many years, some have used the nad5 gene which is not informative enough to confirm certified results in different regions, and others analyzed COX-1 or NAD-1 or both with traditional sequencing methods as sangar sequencing or RFLP, but their limitations stood in the way of achieving satisfactory results and lacked many research methods' inevitable features as the specificity and effectiveness in sequencing multiple reads and took a

relatively long period with high cost, leading to questionable results and maybe treatment mistakes. It's dependent on prior knowledge of informative polymorphic regions to analyze a particular gene with a few kilobases and the expensive costs needed for a huge number of DNA sequence analyses. Still, with the invented technologies such as PCR and next-generation sequencing, more samples can be analyzed in a shorter time, about 2-3 hours.

Scientific evidence could firmly affirm the presence of parasite species in a particular area, and this study provides valuable banners to create a database that would help the competent authorities in the diagnosis and treatment of the disease, likewise answering many outstanding questions, and filling some gaps in understanding the parasite's epidemiological and pathological patterns. To our knowledge, this is the first powerful method that can diagnose echinococcosis by 7 SNPs at the same time using NGS and software analysis in massively parallel sequencing opposite to sangar which requires a larger amount of input DNA, short fragments 300 – 1000 bp are required for sangar sequencing which may degrade after 700 – 900 bases, thus it's one of the reasons why sangar is used to validate NGS results recently. NGS as well is also capable of revealing the presence of two genotypes at the same host "co-infection" regardless if it's human or animal showing two profiles of the same sample in 50 to 50 % of resulting reference and alternative nucleotides, which adding one of NGS advantages and unprecedented approach compared to sangar or other sequencing methods which produce limited throughputs, for NGS sequencing sample preparation method applies sequence based on one molecule. In contrast, sangar or RFLP uses a pool product that prevents the selection of two types in pool sequences and as a result, the sample may fail. It might be the first research capable of identifying all samples directly by two PCR steps and NGS sequencing, with high-quality outputs and a low margin of errors.

For Palestine, the high consumption of the livestock industry may explain the high incidence of echinococcosis, along with many other factors that could affect the variation of species, genotypes, and host susceptibility, which justifies the importance of genetic studies. The reasons beyond species variation could refer to many factors, such as the geographical distribution, and the nature of the parasite itself which displays a wide diversity of its genome, as well as the variation of the host is a contributor to that genomic diversity. Disease spreading might be attributed to cultural awareness following thoughts and beliefs that may support the parasite life cycle, high dependence on the livestock industry, illegal slaughtering of some abattoirs, poor hygiene habits, the abundance of homeless dogs' packs which are fundamental in the parasite's life cycle and absence of periodic checkups for humans and animals in rural areas.

Some recommendations would help reduce disease progression, the highest priority is constricting the risk factors, followed by the general safety instructions. All categories are involved in this cycle, and collaboration efforts are required by everyone, especially the butchers and public health professionals in the first place reaching the public. CE is a preventable disease through breaking the dog-livestock cycle, following some recommended points. (1) First of all, public education campaigns have a crucial role in controlling the disease, depending on the ideas and beliefs of individuals that control the whole society's behavior. (2) public health and hygiene awareness which may reduce transmission of pathogens in general (3) Periodic deworming of dogs with anthelmintic drug praziquantel at least four times per year, (4) Improved hygiene in the slaughtering of livestock (including the proper destruction of parasite-infected offal and avoiding feeding to dogs, also inspecting meat to make sure that it's free of the cyst), (5) Sheep vaccination using *E. granulosus* recombinant antigen (**EG95**) vaccine which offers high protection against the

parasite and decreasing in livestock industry loss. A program composed of vaccination of cattles, deworming of domestic canids, and weeding out the older sheep could dispose of the disease among humans in less than 10 years. Perception of the parasite's life cycle helps grasp how to deal with the pandemic and achieve good control programs(Amarir et al., 2021).

This study provided a precise and particular method to study *E. granulosus* species in depth, NGS facilitates epidemiological studies and saves time simultaneously, we had the opportunity to study the *E granulosus* species and distinguish G1 and G3 genotypes in Palestine using NGS based on seven informative regions of the parasite's mitochondrial genes together using human and animal cyst tissue samples at high incidence levels for the first time referring to G1 genotype as a standard base to our analysis on the contrary of previous studies, that used only few subunits of the mitochondrial genes or some of the mentioned SNPs to distinguish the parasite's species. Previous research conducted a partial sequence of the mitochondrial gene and yielded results in small percentages using conventional methods which required many steps that may take several days or months(Adwan et al., 2013, Beiromvand et al., 2011), while this recent study has confirmed certain results of a high incidence of G3 genotype in Palestine in a relatively short time with minimal flaws.

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Appendices:

Appendix 1. DNA extraction from a human and animal tissue sample standard protocol according to the NucleoSpin® tissue kit

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Bits of advice to facilitate the lab work:
Prepare the water bath incubator at 56ºC, and make sure to prepare the proteinase K and Buffer B5 according to manufacturer instructions before starting the experiment.

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1. After preparing the tissue (pre-treatment steps), and thawing the samples at room temperature, 200 µL of Buffer B3 was added with vortex followed by incubation at 70ºC for 10 min.

2. 210 µL of ethanol was then added and vortexed vigorously.

3. All of the sample precipitates were loaded to a NucleoSpin® tissue column within a collection tube and centrifuged by Biofuge pico® machine at 11,000 x g for 1 min. The collection tube was then discarded and replaced with a new one.

4. 500 µL of Buffer BW was added for the first wash and centrifuged for 2 min. At this point, the flow was discarded and kept the same column baked.

5. For the second wash, 600 µL of Buffer B5 was added and centrifuged for 2 min. and again the flow was discarded, and the same column was kept.

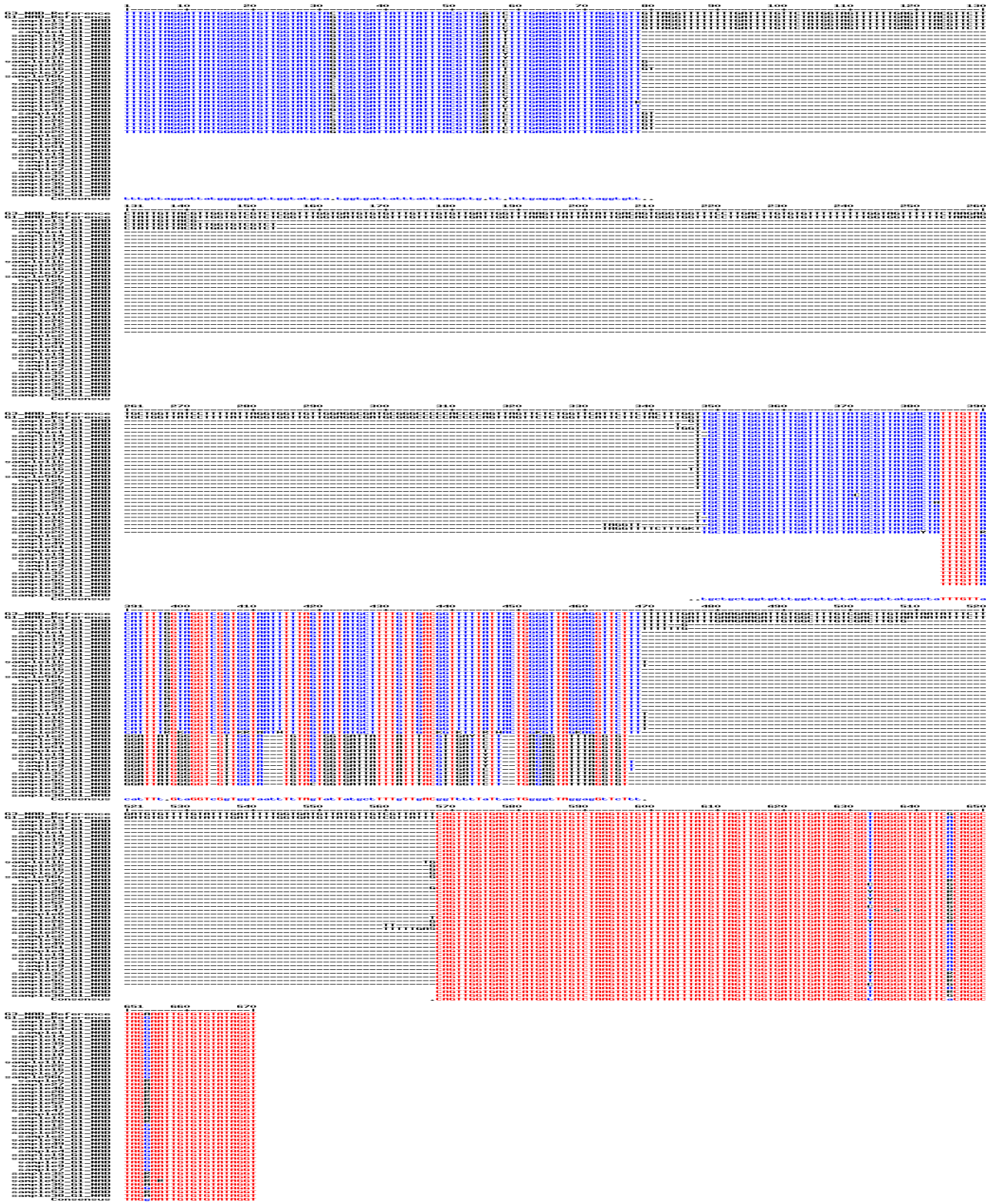
6. Centrifuging for 3 min was done then to get rid of the residual ethanol.

7. The NucleoSpin column was transferred into a microcentrifuge tube and 60 µL of Buffer BE (elution buffer) was then added to elute the DNA, followed by incubation for 1 minute at room temperature and centrifuging at 11,000 rpm for 3 min. finally, the tissue column was discarded, but not the microcentrifuge tube which was kept holding the extracted DNA.

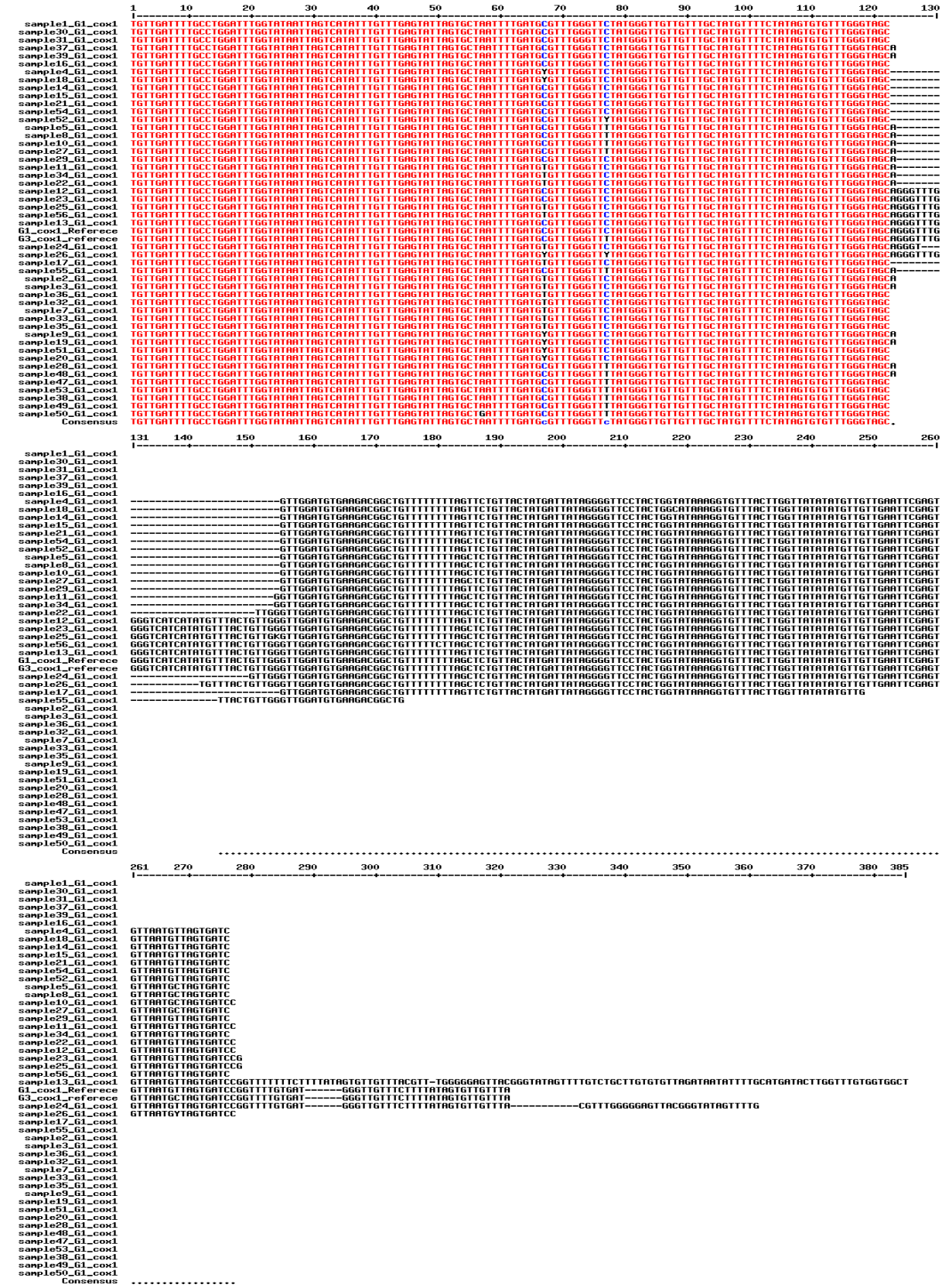
8. After all, samples were stored at -20ºC until needed.

Appendix 2. Alignment of samples identifying SNPs positions for each sample using MultAlin, a conventional method for genotyping analysis.

Appendix 2.1: NAD-1 reference gene results:



Appendix 2.2: COX-1 gene reference results for all sequenced samples:



Appendix 3. Blast of sequenced positive screened samples by Sanger sequencing:

E

Echinococcus granulosus repeat region sequence	Echinococcus granulosus	89.8	323	17%	9e-13	100.00%	269	DQ157697.1
Echinococcus granulosus isolate Q8 EgG1 HaeIII repeat region	Echinococcus granulosus	89.8	172	8%	9e-13	98.08%	178	KX017218.1
Echinococcus granulosus clone DEF.2 repeat region	Echinococcus granulosus	87.9	87.9	4%	3e-12	98.00%	689	KR347166.1

F

Download GenBank Graphics Sort by: E value

Echinococcus granulosus repeat region sequence

Sequence ID: DQ157697.1 **Length:** 269 **Number of Matches:** 4

Range 1: 7 to 54 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
89.8 bits(48)	9e-13	48/48(100%)	0/48(0%)	Plus/Plus

```
Query  100  CGCATGAGGATTACTGACTTGAACGATCACTCCTATTCTAGCATGTGT  147
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7    CGCATGAGGATTACTGACTTGAACGATCACTCCTATTCTAGCATGTGT  54
```

H

Echinococcus granulosus isolate K9 EgG1 HaeIII repeat region	Echinococcus granulosus	143	143	14%	3e-29	97.59%	178	KX017220.1

G

Download GenBank Graphics

Echinococcus granulosus isolate K9 EgG1 HaeIII repeat region

Sequence ID: KX017220.1 **Length:** 178 **Number of Matches:** 1

Range 1: 15 to 97 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
143 bits(77)	3e-29	81/83(98%)	0/83(0%)	Plus/Plus

```
Query  25   GTGGGCCGTGTTGTGGACGTAGTTTCGGCATCTAGTGAAGGAAAAATAAGACATCGGTGC  84
            |||||||||| ||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  15   GTGGGCCGTGCTGTGGAGGTAGTTTCGGCATCTAGTGAAGGAAAAATAAGACATCGGTGC  74

Query  85   GAGCACTCCTTCTCACTCATCTC  107
            |||||||||||||||||||||||
Sbjct  75   GAGCACTCCTTCTCACTCATCTC  97
```

الكشف الجزيئي والتنميط الجيني لداء الكيسات المائية من الأكياس المائية في الماشية المذبوحة في الضفة الغربية، فلسطين.

إعداد: ولاء حابس محمد يونس.

المشرفون: د. سهير عريقات، د. عبد المجيد ناصر الدين.

الملخص

الخلفية: على الرغم من انتشار داء الكيسات المائية على نطاق واسع، إلا أن هناك فجوة كبيرة في فهم الأنماط الجينية المحددة لطفيلي الإكينوكوكس الموجودة في مناطق مختلفة، بما في ذلك الضفة الغربية في فلسطين. هذا النقص في المعرفة يعيق فعالية إدارة المرض واستراتيجيات مكافحته. يُعد تحديد الطفيليات التي تصيب العوائل الوسيطة والتنميط الجيني لها أمرًا بالغ الأهمية للمراقبة الوبائية وآثارها على الصحة العامة، من خلال توعية السلطات الصحية وصانعي السياسات لتطبيق تدابير مكافحة فعالة بناءً على البيانات الوبائية المحلية. هدفت هذه الدراسة إلى تحديد وتحديد النمط الجيني لطفيليات الإكينوكوكس المسببة لداء المشوكات الكيسي (CE) في الضفة الغربية بفلسطين، مع التركيز بشكل خاص على العوائل الوسيطة (IMHs) مثل الأغنام والماعز.

المواد والطرق: جُمعت 54 عينة نسيجية من الماشية في الضفة الغربية، 52 عينة نسيجية من الأغنام من مسالخ مختلفة في مناطق معروفة بارتفاع معدل الإصابة بداء المشوكات الكيسي في الضفة الغربية، تتكون من أنسجة كيسات مائية، عُزلت من الأعضاء المصابة أثناء فحص العائل الوسيط، وعينتان من أنسجة كيسيات بشرية جُمعت من المستشفيات بعد الجراحة كعوائل وسيطة عرضية. استُخلص الحمض النووي (DNA) وضخّم بواسطة تفاعل البوليميراز المتسلسل (PCR) لضمان جودة عالية للحمض النووي الجينومي للتسلسل باستخدام تسلسل ***EgG1 Hae III*** (266 زوجًا قاعديًا). خضع الحمض النووي المستخرج لتقنية الجيل التالي (NGS)باستخدام مجموعتين من البادئات، مما يسمح بإجراء تحليل جيني شامل. استهداف جميع تعددات النوكليوتيدات المفردة (SNPs) لكلا الجينين الميتوكوندريا في آن واحد COX-1 :(الوحدة الفرعية 1 من أوكسيديز السيتوكروم سي) وNAD-1 (الوحدة الفرعية 1 من نازعة هيدروجين NADH) ، واللذين تم اختيارهما لتنوعهما وفائدتهما في التمييز بين أنواع وأنماط جينية مختلفة من الإكينوكوكس. وأخيرًا، استخدام منصة تحليل استدعاء المتغيرات لتحديد وتنميط التسلسلات الخاصة بالإكينوكوكس من بين آلاف التسلسلات من خلال برنامج المعلوماتية الحيوية الإلكتروني.Galaxy/Europe

النتائج: تم تحديد تسلسل ***EgG1 Hae III*** بطول 266 زوجًا قاعديًا في 98% من العينات. تم تضخيم أجزاء NAD1وCOX-1الخاصة بنجاح وتسلسلها من خلال منصة NGS من 53 عينة من أنسجة الكيس. أُجري تحليل النمط الجيني بنجاح في 84% من العينات المتسلسلة، وكشف أن 58% منها تنتمي إلى النمط الجيني G1و42% منها تنتمي إلى النمط الجيني.G3

الخلاصة: هدفت هذه الدراسة إلى التمييز بين النمطين الجينيين G1 s.s و G3في عزلاتنا بناءً على سبع مناطق معلوماتية مختلفة لأول مرة، أكد استخدام تقنية NGS بمخرجات عالية الجودة هيمنة النمط الجينيG1 ، مع

الإشارة أيضًا إلى وجود كبير للنمط الجيني G3 بين العوائل الوسيطة في المناطق الموبوءة في فلسطين. وبينما تحظى تقنية NGS باعتراف واسع النطاق في الأوساط العلمية، تؤكد نتائجنا على أهمية فهم علم الأوبئة المحلي لداء المشوكات الكيسي. يوفر تحديد كل من النمطين الجينيين G1 و G3في هذه المنطقة بيانات بالغة الأهمية يمكن أن تُثري استراتيجيات الصحة العامة وتدابير المكافحة المستقبلية.

الكلمات المفتاحية: E. granulosus، العائل الوسيط (IMH)، mtDNA (NGS)، SNPs لداء المشوكات الكيسية.