Molecular characterization of fertile hydatid cysts from the liver of the sheep and cows and associated environmental influence factors

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Abstract

The aim of the study is characterizing of hydatid cysts that have been isolated from sheep and cow liver fertile hydatid cysts using mitochondrial cytochrome c oxidase subunit 1(cox1) gene. DNA samples of nineteen sheep and one cow were extracted from protoscoleces and germinal layers of the parasites in Koya city-Erbil, Iraq, using specific extraction procedures. Mitochondrial cox1 gene region was amplified by polymerase chain reaction (PCR), and the expected gene sizes were confirmed by gel electrophoresis. All DNA isolates were then sequenced. Nucleotide sequence alignments were then performed to verify the sequenced isolated according to the database, which showed that all samples were belonging to the (G1) sheep strain. Phylogenetic analysis was also carried out for the sequenced isolated to find out the highest similarities with closest organisms to *E. granulosus'* conserved gene and to reveal sharing common ancestor, which has been confirmed. Electrocatalytic reduction of DNA where detected through applying cyclic-voltammetry technique, which referred to the environmentally strong protection features of these strains against any effects of external factors, such as heavy metals and has revealed the secret behind the potent preservation of the DNA structure of this parasite from being affect by mutations, or alterations, along the different lineages over a long period of time.

Keywords: Hydatid cysts, Cytochrome C oxidase, Sheep strain, Phylogenetics, Cyclic-voltammetry

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التوصيف الجزيئي للأكياس العدرية الخصبة المعزولة من كبد الأغنام والأبقار وعوامل التأثير البيئي المرتبطة بها

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الخلاصة

الهدف من الدراسة هو توصيف النمط الوراثي للأكياس العدرية الخصبة المعزولة من الأكباد للأغنام والأبقار باستخدام المورثة سيتوكروم سي أوكسيديز الوحدة الفرعية ١ الموجودة في ميتوكوندريا الطفيلي. تم استخراج عشرين عينة من الحمض النووي (تسعة عشر من الأغنام وواحدة من الأبقار) من الطفيليات اليافعة والطبقات الجرثومية للأكياس العدرية الخصبة للطفيليات من مناطق مختلفة في مدينة كوية - أربيل، العراق، باستخدام إجراءات استخراج محددة. تم تضخيم منطقة جينة الميتوكوندريا بواسطة تفاعل البلمرة المتسلسل، وتم التأكد من أحجام الجينات المتوقعة بواسطة الرحلان الكهربائي الهلامي. تم تحديد التسلسل الوراثي لجميع عزلات الحمض النووي باستخدام تقنية تتابع سلسلة قواعد الدنا. بعد ذلك تم إجراء محاذاة تسلسل النيوكليوتيدات للتحقق من التسلسل الوراثي للجينات المعزولة وفقا لقاعدة البيانات، والتي أظهرت أن جميع العينات تنتمي إلى سلالة الأغنام جي ١. تم إجراء تحليل النشوء والتطور أيضًا للعزل التسلسلي لاكتشاف التشابه مع الكائنات الحية والكشف عن مشاركة سلف مشترك، وهو ما تم تأكيده. التحلل الكهربائي للحمض النووي تم اكتشافه من خلال تطبيق تقنية

قياس الجهد الدوري، والتي تشير إلى ميزات الحماية القوية بيئياً لهذه السلالات ضد أي آثار لعوامل خارجية، مثل المعادن الثقيلة وكشفت السر وراء الحفاظ القوي على بنية الحمض النووي لهذا الطفيلي من التعرض للطفرات، أو التعديلات، على طول الأنساب المختلفة على مدى فترة طويلة من الزمن.

Introduction

Echinococcosis is a cosmopolitan zoonosis caused by the adult or larval stages of cestoda belonging to the genus *Echinococcus granulosus*, however larval infection (hydatid disease, hydatidosis) is caused by the larval stages of the parasite and characterized by long-term growth of tissue-invading metacestode (hydatid) cysts in the intermediate host, such as sheep and cow. Hydatidosis has worldwide medical and economic impacts and considered as a major public health importance, which cause human cystic echinococcosis that are responsible for almost all the human and animal burden (CE) (1).

Applications of molecular tools for genetic characterization of *E. granulosus* are significant to understanding the *E. granulosus* genotypes has had a significant impact on the phenotypic characteristics, host specificity, antigenicity, transmission dynamics, infection route, pathology, control, sensitivity to chemotherapeutic agent and vaccine development strategies (2). Lately more accurate molecular techniques are used for detecting species and strain of *E.granulosus*. Based on nucleotide sequence analysis of the mitochondrial cytochrome oxidase subunit 1 (CO1), dehydrogenase subunit 1 (ND1) genes and intra transcribed spacer 1 (ITS1) (3). Ten genotypes have been described in a world isolated from sheep, pigs, cattle, horses, camels, goats, and human (4).

Mitochondrial DNA (mtDNA) has more power than nuclear DNA in reforming phylogenetic relationships among closely related species, this is due to the rapid sequence evolution and large data sets derived from mitochondrial genomes that have the potential for resolving problematic issues in taxonomy. According to the stage, size, localization and complications of the cysts, hydatidosis can be treated (5). Presently, four treatment modalities are in use for hydatid disease: chemotherapy, percutaneous aspiration, injection and reaspiration (PAIR) and surgery (6). Definitive hosts do not normally possess any symptoms though being infected by adult worms, while the larval stage of *Echinococcus granulosus* prompts significant pathology in the intermediate host. Small cysts may stay asymptomatic indefinitely, but the large cyst cause symptoms by compressing adjacent structures. Most *E. granulosus* cysts are 1-10 cm in diameter when they are discovered, but some may eventually reach more than 20 cm (7).

Approximately 60%-70% of hydatid cysts takes place in the liver and 20%- 25% followed in the lungs. Bones, kidneys, spleen, muscles, CNS and behind the eye is the other organs infected by cysts (8). However, symptoms of hydatid disease can product from the release of antigenic material and secondary immunological reactions that develop following cyst rupture. Fever and acute hypersensitivity reactions ranging from urticaria and wheezing to life-threatening anaphylaxis may be the principal manifestations. While allergic episodes may develop after cyst rupture, fatal anaphylaxis is uncommon (8). Cystic echinococcosis can be controlled through numerous blocking measures that break the life cycle between the definitive and intermediate hosts; however, the most operative means to resistor hydatid disease in humans and eliminate the consequences of *Echinococcus* infections in livestock is through the broad range education of people living in endemic regions (9). Contemporary phylogenetic hypotheses have been largely based on data derived from mitochondrial sequences, as mtDNA provides useful genetic markers, whereas an alternative source of phylogenetic characters from the nuclear genome has not received much attention (10).

Heavy metals pollution is a global issue, though severity and levels of pollution differs from place to place. At least 20 metals are classified as toxic with half of them emitted into environment in concentrations that pose great risks to human health. The common heavy metals that have been identified in polluted water include arsenic, copper, cadmium, lead, chromium, nickel mercury and zinc (11).

The best indication supporting the hypothesis of the oxidative nature of metal-induced genotoxic damage is provided by the wide spectrum of nucleobase products typical for the oxygen attack on DNA in cultured cells and animals exposed to carcinogenic metals. The genetic material inside the cell is damage though the interaction between the DNA structure and its pattern. Genotoxic substances are the main behind this damage of the genetic material (12).

The proper mechanism by which heavy metals damage the DNA of the body comprised of heavy metals having greater atomic size within cell molecular structure will replace minerals in the near future (13).

The aim of this study is to determine the molecular characterization of larval stage of *E. granulosus* and identification of common strain in Erbil governorate, Kurdistan region, Iraq, in addition to phylogenetic analysis for the sequenced isolates, to find out the highest similarity of conserved gene with homologue species, and estimate the electrocatalytic reduction of DNA by using cyclic voltammetry technique.

Materials and methods

Sample collection

In this study, 19 fertile hydatid cysts of sheep and 1 from cow were collected from slaughtered in abattoirs located in Koya, Erbil, Iraq. The organ infected with hydatid cysts were placed in a clean plastic sac and transported to the research laboratory of the (Science and Health Research Center of Koya University) in a cool box contained ice.

Viability of protoscoleces

0.05% methylene blue solution was prepared by dissolving 0.05 gm of methylene blue dye in 100 ml of 0.15 M PBS PH 7.4 (8 gm of sodium chloride (NaCl), 0.2 gm of potassium chloride (KCl), 1.44 gm of dibasic sodium phosphate (Na_2HPO_4) and 0.24 gm of potassium di-hydrogen phosphate (KH_2PO_4) in 1000 ml distilled water). Methylene blue exclusion test was achieved by mixing 0.02 ml of methylene blue solution with an equal volume of protoscoleces suspension and examined microscopically (14).

DNA extraction

Genomic DNA was extracted from twenty samples of protoscoleces and germinal layers using commercial DNA extraction kit PrimePrep™ Genomic DNA Extraction Kit from tissue. According to the manufacturer s instructions and then stored at -20˚C until PCR amplification.

Polymerase chain reaction

For the purpose of amplification of the Cox1 gene fragment, the following primers were designed and used; (5' -TCT CTG CAT TTG GCT GGT GT - 3') (5' - CCC TGC TAC CCA AAC ACA CT- 3'), as forward and reverse respectively. Fifty microliter reaction were used and amplified by PCR under the following temperature conditions: initial denaturation 94°C for 5 min, and then denaturation 94°C for 30 sec, annealing 56°C for 30 sec, extension 72°C for 30 sec, in 30 cycles and final extension 72°C for 5 minutes. PCR products were electrophoresed on 0.5% agarose gel after staining with ethidium bromide and then Cox1 gene was seen under UV light using transilluminator (15).

Sequencing and phylogenetic analysis

20µl of PCR products and 20µl of (10 Pmol) primers for each sample were transferred to 1.5 ml tube for sequencing, and sent to company in Korea for DNA sequencing by AB DNA sequencing system. The chromatograms were analyzed and the nucleotide sequences obtained were aligned using the ClustalW method of the program MEGA 7 (megasoftware.net), using the sequences of the different genotypes of *E. granulosus* deposited in GeneBank as reference. However phylogenetic analyses of the sequences data were completed using Cox1 sequences and phylogeny tree was drawn using sequences obtained in this study as well as reference sequences of all described *E. granulosus* genotypes by MEGA 7 software.

Electrode and apparatus

The conventional detection >1.0ml using SPE glassy carbon electrode 61208110 (Metrohm, Spain). The small sample detection <1.0ml using platinum electrodes (designed and made by our laboratory). The platinum electrodes including a working electrode (platinum electrode), an opposing electrode (platinum electrode), and a reference electrode (Ag-AgCl electrode) used for cyclic voltammetry measurement (16).

Results

Subject selection

The total number of samples included in this study was 20. Nineteen of them isolated from sheep and one case were exist in cow, from Koya slaughterhouse (Figure 1). Different organs in these animals were infected with hydatidosis, the number of cases with liver hydatidosis were 12, followed by lungs infections at 8 cases of hydatidosis.

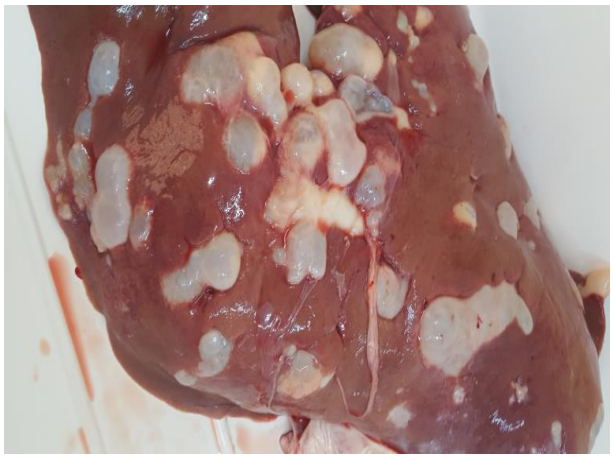

Figure 1: Clarifies infected sheep liver with hydatid cysts.

Cyst integrity

The numbers of intact cyst were 100% whereas the number of rupture cyst were 0%.

Size of cyst

Most infected animals with hydatidosis are large cysts involvement 5 cm in diameter, and these cysts were seen more pronounced in liver than lung the diameters of cysts that generally increase are variable.

Methylene blue test

Protoscoleces viability was assessed through applying Methylene blue test, which assured under light microscope (Figure 2).

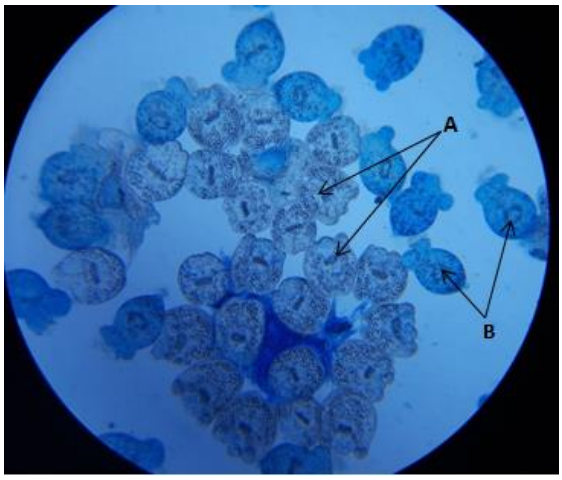

Figure 2: Methylene blue test for the protoscoleces viability. A. live protoscoleces did not retain the stain. B. dead protoscoleces took blue color, 40X.

Amplification of gene by PCR and Sequencing analysis of CO1 gene

PCR amplification was done successfully for isolates hydatid cyst. After electrophoresis of PCR product, 450 bp bands were seen in all samples under UV light clearly shown in (Figures 3-5). G1 genotypes were identified from the isolates which out of 19 sheep and 1 cow hydatid cyst. The results of this study indicate that the predominant genotype of *E. granulosus* in Iraq was G1 sheep strain. The multiple sequence alignment was done by using Bioedit (DNA analysis program) and compared with previously reported references of the gene.

The results revealed that 99.15% and 100.00% of the alignment outcomes were identical with common sheep strain G1 genotype.

Cyclic voltammetry

The cyclic voltammetry technique was applied for the detection of DNA interaction with specific electroactive materials. Firstly, the DNA was extracted from specimens and amplified through (PCR). However, there is no clear peaks of ipA/ipC were the real-time measured by special designed electrode, so the DNA has lack quantitative detected as shown in (Figure 6).

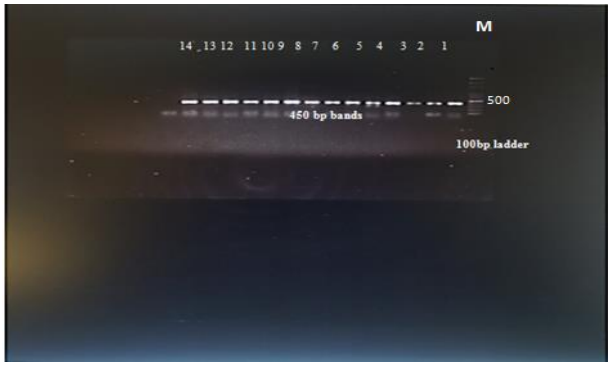

Figure 3: Amplified cytochrome C oxidase subunit 1 (CO1) gene of hydatid cyst DNA isolates from protoscoleces, Line 1 Ladder (100bp), (Line 2 cow liver) (Lines 3-14 sheep liver) at 450bp.

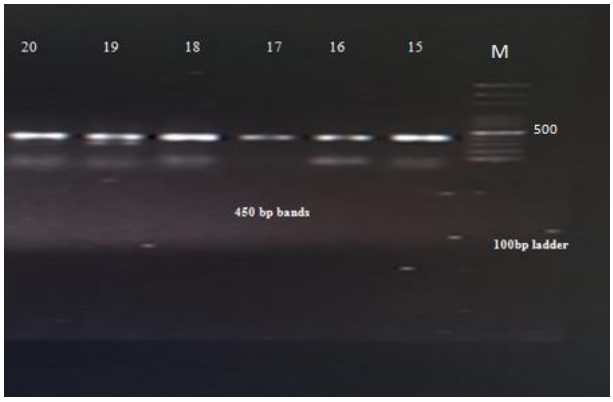

Figure 4: Amplified cytochrome C oxidase subunit 1 (CO1) gene of hydatid cyst DNA isolates from protoscoleces, Line 1 Ladder (100bp), (Lines 15-20 sheep samples) at 450bp.

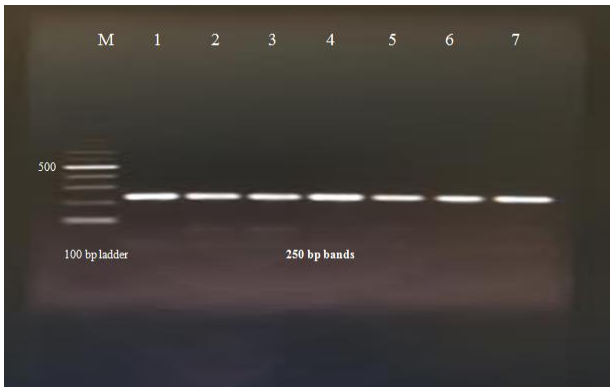

Figure 5: Amplified cytochrome C oxidase subunit 1 (CO1) gene of hydatid cyst DNA isolates from germinal layer, Line 1 Ladder (100bp), (Lines 1-7 sheep samples) at 250bp.

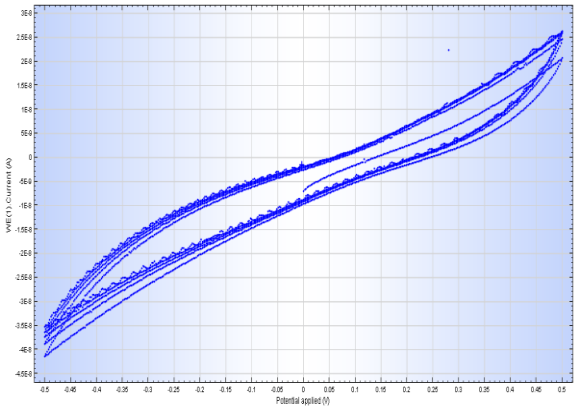

Figure 6: Amplified DNA samples were combined with positive dye of characterized Methylene blue dyes in the cyclic voltammetry (methylene blue in 1.0 mg/L-1) that leads to a redox couple peaks current (ipA/ipC). No clear peaks of ipA/ipC of DNA interaction were detected in the real-time by special designed electrode.

Discussion

Genotype G1 is a prevailing genotype found in world livestock (17). These finding suggests that the sheep-dog cycle occur in this region. However, genotyping of sheep hydatid cysts using sequencing with cox1 gene, G1 is described as predominant genotype in various countries such as Peru (18), Egypt (19) China, Turkey, Italy and Tunisia that are consistent with this study (20-22) and with highly remarkable corresponding to north African and Mediterranean countries isolates (23). Genotype of *E. granulosus*, mitochondrial genes are much better than other genome sources for *E. granulosus* genotyping. Data obtained from mitochondrial DNA can help researchers to solve parasite taxonomy problems (24). The molecular identification of *E. granulosus* has a clinical importance value for control and prevention (25). Hama *et al.* (26) support the current study which revealed that the CO1 has the ability and good efficacy to determine a strain and genetic variation.

The present-study findings agree with (26-28) they stated that the sheep strain G1 is the common strain in Iraq, which has an ability to infect most intermediate hosts including sheep, cattle, goat, and human. In these poorer regions, dogs are often fed with livestock viscera that may be infected with the parasite. This activity alone could be sufficient to propagate the current endemic state. From the results above, it can be indicated that the prominent circulation of the common sheep genotype G1 was prevalent in high endemic areas of Erbil. These results are in agreement with the findings of (29) as the most common type that is also quite prevalent among intermediate hosts.

The result of cyclic voltammetry was showed no clear peaks of ipA/ipC were the real-time measured by special designed electrode, so the DNA has lack quantitative detection. This could be due to the fact that the DNA structure of hydatid cysts are resistant and possess significant protections against environmental factors and decreases the chances of being undergo modification in the structure of DNA and eventually mutation. The DNA amplification products of PCR could be combined with positive dye such as methylene blue which leading to a redox couple peaks current (ipA/ipC) of the cyclic voltammetry (30). The outcomes of the cyclic voltammetry DNA analysis are also suggesting an indication of the protected features of the amplified gene against external harmful factors which can refer to their conserved characteristics along consequence lineages. Cyclic voltammetry has been highlighted that electrochemical DNA probe had been applied for the quick and sensitive detection of *Salmonella* (31). Recently, they have established an electrochemical method, real-time resistance measurement, for amplification and detection of DNA (32). Likened with the resistance of real-time measurement, the cyclic voltammetry technique is more precise and more suitable for DNA detection on gene chip. The electrochemical method is fast and cheap and could be made measurements under a diversity of experimental environments (33). The electrocatalytic reduction of DNA has been an achieved at a Glassy carbon electrode using cyclic voltammetry (34). The composition of multiple electroactive materials in the cyclic voltammetry at electrodes has been tested, where zones of one highly active material are distributed over a substrate is investigated by simulation (35). The CV electrode has led to detect very small electrical signals, and therefore it was attempted for DNA sensor applications (36).

According to Wang *et al.* (37) only a few works on the direct electrochemical behavior of DNA at solid electrodes were reported. The electroactive groups for the electrochemical oxidation of DNA at GCE, the sensitivity of DNA detection in these works is not satisfactory and the shape of cyclic voltammograms is also very poor. However, many objects have focus on the reduction of DNA like mercury and gold forming DNA binding complex and produce a clear peak (38). The cyclic voltammetry method in comparison with the PCR is more accuracy, sensitivity and specificity (38).

Conclusion

The study confirmed that the sheep strain G1 genotype was the common strain in Erbil, Iraq. G1 genotype is an endemic in Erbil, Iraq and the actual source of infection in animals, mtDNA extraction from protoscoleces, yielded large amount of mtDNA extracted from germinal layer. Phylogenetic analysis detected that the sequenced isolated

from *E. granulosus* share the highest similarity with other organisms. Cyclic voltammetry shows no clear peaks of ipA/ipC were the real-time measured by special designed electrode confirming the highly conserved gene properties and their resistance to interaction, and consequently to mutation.

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Conflict of interest

I am certifying that there are no financial and personal relationships with other people or organizations which can influence or bias their work and declare that there is no any conflict of interest.

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