MOLECULAR SEQUANCING AND PHYLOGENIC ANALYSIS TO VIRULENCE *nmuc-1* GENE IN VISCERAL LARVAE MIGRANCE

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ABSTRACT

 Toxocariasis is a zoonotic parasitic disease caused by *Toxocara canis* infected egg. Larval stage of this parasite has ability to migrate through intestinal wall and invade all body organs causing a visceral larvae migrant (VLM) syndrome. Diagnosis of VLM is problematic; there were no accurate laboratory test that reveals the presence of larvae infection in paratenic hosts (human, ruminants or poultry). The eggs were isolated from adult *T. canis* uteri and cultured in 0.2M H2S04 solution for embryonation, mice were experimentally infected with emberyonated eggs. Many hisopathological changes detected in heart and kidney tissues of infected mice but it could not detected encysted larvae compared with molecular detection which confirmed infection within first three day post infection in tissue with accurate diagnosis for the first time in Iraq depend in detected virulence *nmuc-1* gene. Phylogenic tree analyses mounted a low genetic variation (0.2) among Iraqi isolate and all other comparison isolates. In conclusion our result indicated that molecular method could diagnosis *T. canis* larvae infection in any meat or meat products of local or imported from inside or outside Iraq country and used as an accurate microbiological laboratory test used routinely in government laboratories.

Keywords: *Toxocara*, histopathology, heart and kidnys tissues.

التسلسل الجزيئي وتحليل الشجرة التطوري لجين الضراوة *nmuc-1* المتسبب بمتلازمة هجرة اليرقات الحشوية

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مدرس

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المستخلص

داء السهميات مرض ناجم عن الاصابة ببيوض طفيلي *Toxocara canis* . تتميزمرحلة الاصابة بالطور اليرقي لهذا الطفيلي بقدرته على الهجرة عبر جدار الأمعاء وغزو جميع أعضاء الجسم مسببة متلازمة اليرقات الحشوية المهاجرة(VLM) ، لا يوجد تشخيص لهذه المتلازمة او اختبار دقيق يكشف وجود يرقات الطفيلي في جسم المضيف (الإنسان أو المجترات أو الدواجن). تم عزل البيوض من رحم الطور البالغ للطفيلي *T. canis* وتنميتها بمحلول 0.2M H2S04 لغرض الحصول على بيوض مخصبة وإصابة الفئران تجريبيا . تم تشخيص التغييرات النسيجية في عضوي القلب والكلى في أنسجة الفئران المصابة بالرغم من عدم امكانية كشف الطور اليرقي للطفيلي مقارنة مع نتائج الفحص الجزيئي للانسجة باستخدام تفاعل البلمرة المتسلسل (PCR) الذي أكد التشخيص المبكر للطور اليرقي للطفيلي اعتمادا على تضخيم جين *nmuc-1* وخلال أول ثلاثة أيام بعد الاصابة ، لأول مرة في العراق. اكدت نتائج شجرة التطور بوجود تباين جيني منخفض (0.2) بين العزلة العراقية وجميع عزلات المقارنة الأخرى ، كما وأشارت النتائج إلى أن التشخيص الجزيئي يمكن أن يكشف عن وجود عدوى اليرقات *T. canis* في عينات اللحوم أو منتجات اللحوم المحلية أو المستوردة من خارج العراق واستخدامه كاختبار دقيق يستخدم بشكل روتيني في مختبرات المستشفيات الحكومية.

كلمات مفتاحية: *Toxocara* ،التشريح المرضي، انسجة القلب و الكليتين.

INTRODUCTION

Toxocara canis remains a problem throughout the world because it causing multisystem zoonotic disease in the paratenic hosts such as humans, ruminants, poultry, rabbit and rodents (8). Human infection occurred accidental by the ingested contaminated soil, food, water or unwashed vegetables with *T. canis* emberyonated eggs which lay on dog feces (dirt); as well as through ingestion of *T. canis* encysted larvae present in undercooked ruminants or poultry liver or meat as a paratenic hosts (26, 21 and 1), leading to many complications due to the migration of *T. canis* larvae to all body organs and invade tissue like liver, lungs or the nervous system (5, 19 and 16). Stool examination for *T. canis* eggs and larvae not useful because larvae not maturated in paratenic host like human and rabbit (17 and 15). Infective larvae (L2) have ability to produce *Toxocara* Excretory/Secretory proteins such as (MUC-120) that help it to penetrate host intestinal wall then invade and migrate to widely various tissues including the liver, lung (10) musculature and CNS, remain on arrested stage without development to adult stage while most infections are asymptomatic but manifests in humans causing the well-characterized syndrome of, ocular larva migrans (OLM), visceral larva migrans (VLM), neurotoxocariasis (NT) and/or covert toxocariasis (CT) (19). The protein MUC-120 expressed by *nmuc-1* gene, surface coat glycoprotein protein (29). *T. canis* larvae could invade host immune system by surface coat protein MUC-120 which is released in response to binding by host antibody molecules or effector cells (31). Positive seroprevalence rate of Toxocariasis in Iraq recorded (27.27%) at child age and (23.33%) in adult people (9). Some degree of antibody cross-reactivity between *T. canis* Excretory/Secretory antigens and proteins excreted by other nematodes lead to false positive results, such as *Trichuris trichiura* (4), *Ascars suum* (6) and hookworm (25). A modern laboratory assay should be used to provide an accurate diagnosis of Toxocaraisis (VLM) infection since no accurate laboratory test that reveals the presence of larvae infection in all paratenic hosts.

MATERIALS AND METHODS

Parasites Samples Collection: Therty two *T. canis* adult worms were obtained from puppies stool naturally infected with *T. canis* after treatment with albendazole. The unemberyonated egg isolated from females uteruses to 0.2M H_2SO_4 solution for embryonation, An experimental infection which involved two mice groups; The first included 12 mice experimentally infected with 1500 embryonated eggs, and the second, control group, 3 mice inoculated distal water. Mice were killed at 2, 7, 11, 18, 24 and 32 day post infection (DPI), two mice each day, small portions from heart and kidny tissues collected in 0.4% HCl for up to 12 h at 37°C, about 3 ml of the sediment were centrifuged for 5 min at 2500 rpm and washed with distilled water to remove acid and stored in -4°C until required for DNA extraction

Histopathological Examinations

Small portion from heart and kidney tissues of infected mice with *T. canis* emberynated eggs transferred to 10% formalin solution 24 h for stained with Hematoxylene and Eosin stain (18).
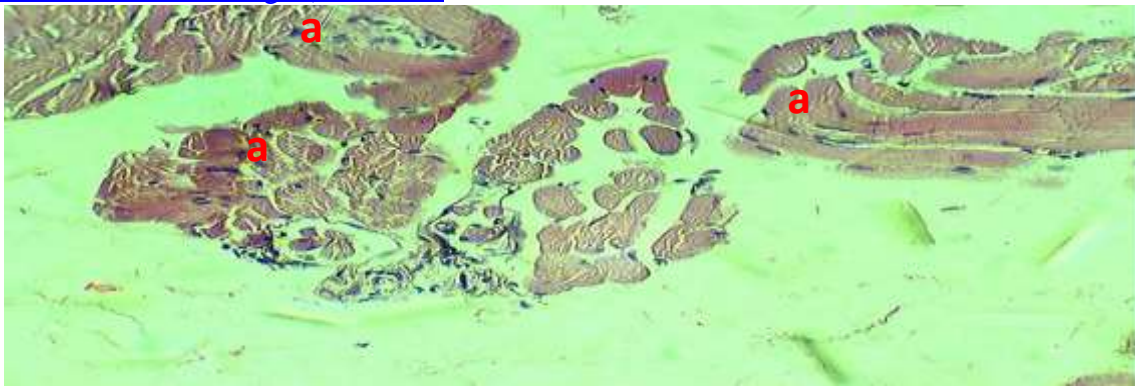
Genomic DNA isolation protocol

Total genomic DNA were extracted from Heart and kidney tissue by grinded for 3 min using a mechanical grinder and was subsequently purified using the commercially available DNA mini Kit (Promega, USA) according to the manufacturer's instructions. DNA concentration and purity were determined by using Nano Drop (Thermo scientific).

Amplification of *T. canis* larvae DNA by conventional PCR

Conventional Polymerase Chain Reacttion (PCR) was performed on all DNA samples to amplify a fragment of *nmuc-1* gene by the designed new specific oligonucleotide primer pair FR5´-TCTGCGTTGCTACACCACAA-3´ and RE5´-AAGTTTGCACAGTCGTTGGC-3´ for each one using purified larvae DNA as a template. PCR final reaction volume 20μL was performed including Master Mix (Bioneer, Korea); 10 pmol of each primer with 1 μL, 3 μL template DNA and 15μL Nuclease-free water. The temperature profile was one cycle of 94° C for 5min (primary denaturation), followed by 40 cycles of 94° C for 30sec

(denaturation), 58°C for 40sec (annealing), and 72°C for 30sec (extension), and a final extension 72°C for 5 min. A sample containing water instead of template DNA was included in each run as a negative control. PCR products were analyzed by electrophoresis on a 1.2% agarose gel and photographed under an ultraviolet transilluminator (20).=

Phylogenetic analyses: forward and reverse sequences from each PCR product were assembled using AccuPrep^w PCR purification Kit (INTRON). Phylogenetic tree carried out employing (Mega 6) Neighbour Joining method to find out phylogeneic relationship with other sequences deposited in NCBI (http://www.ncbi.nlm.nih.gov)GenBank.

RESULTS AND DISCUSSION
Histopathological Changes in Heart and Kidney Tissues:
Microscopic Appearance of Heart Tissue in Infected Mice
The infections appeared earlier on infected mice with *T. canis* empryonated eggs, histological changes observed in heart tissue of infected mice characterized by inflammatory reactions were the heart section showed moderate infiltration of mononuclear cells mainly lymphocyte and macrophage filtration between cardiac muscles as clarifed in figure 1.

Figure 1. Histopathological section in heart tissue of infected mice, showed (a) moderate infiltration of mononuclear cells. (H and E stain, 20X).

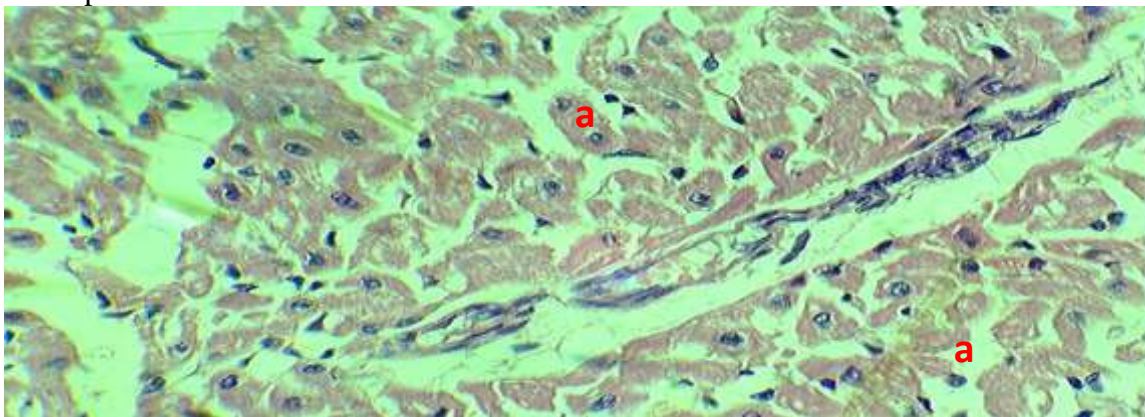Other section in heart tissue showed the vasculature between bundles of cardiac tissue appeared peri and intra infiltration of mononuclear cells mainly the macrophages as clarified in figure 2.
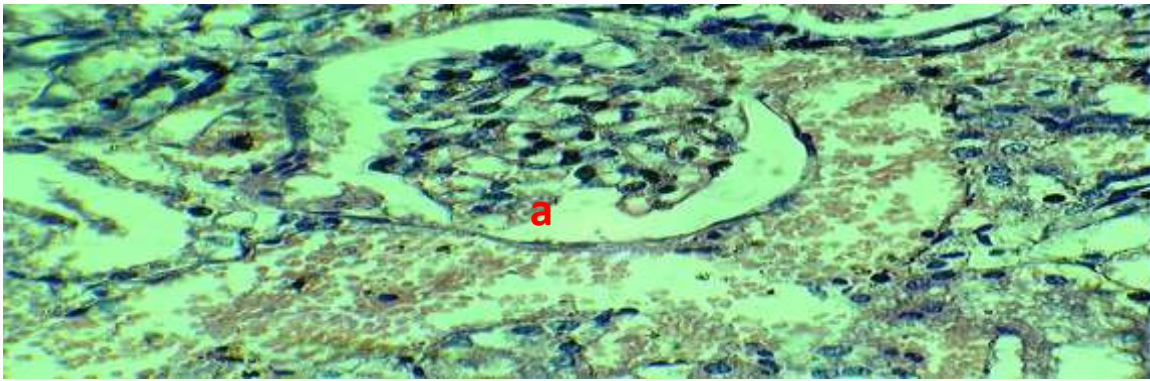

Figure 2. Histopathological section in heart tissue of infected mice, showed vasculature between bundles of cardiac tissue appeared peri and (a) intra infiltration of MNCs. (H and E stain, 40X).

**Microscopic appearance of kidney tissues in infected Mice**
The lesion of renal tissue which infected with embryonated *T. canis* eggs investigated moderate pathological lesion characterized by glomerular endothelial degeneration(vacuolar degeneration) with few infiltration of mononuclear cells (mononuclie , lymphocyte and macrophage cells), as demonstrated in figure 3
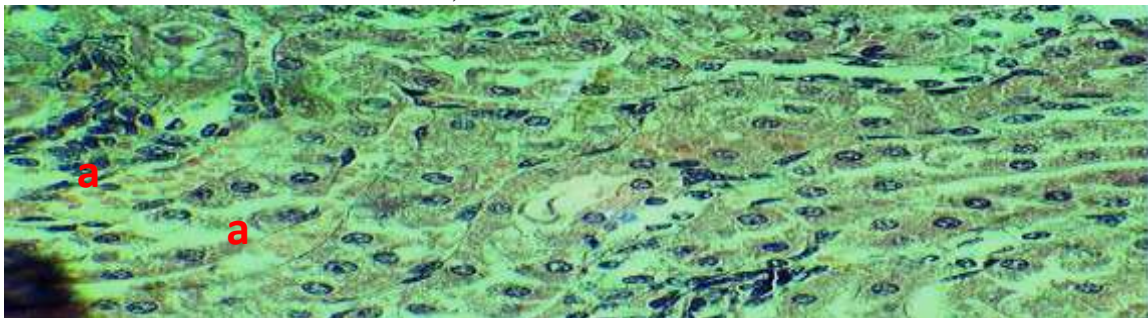
Figure 3. Histopathological section in Kidney tissue of infected mice showed (a) moderate renal tubules degenerative changes (H and E stain, 40X).

The infection also demonstrated enlarged glomeruli due to congested capillaries and infiltrated with MNCs also congestion of the renal blood vessels as clarified in figure 4.
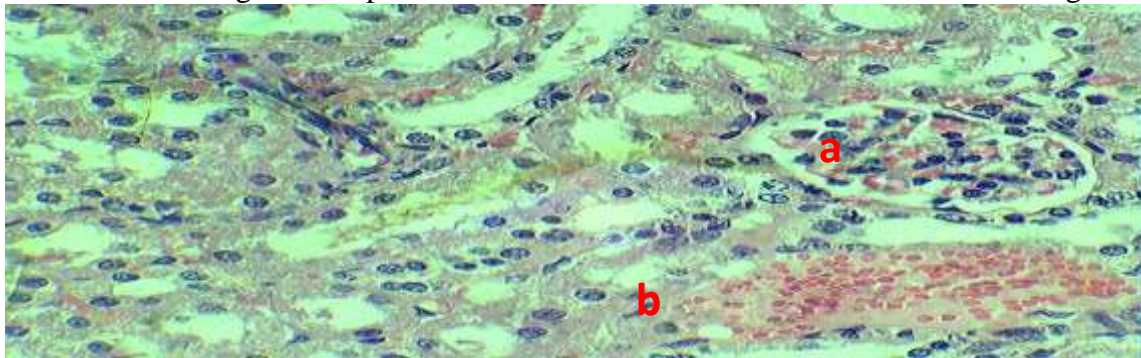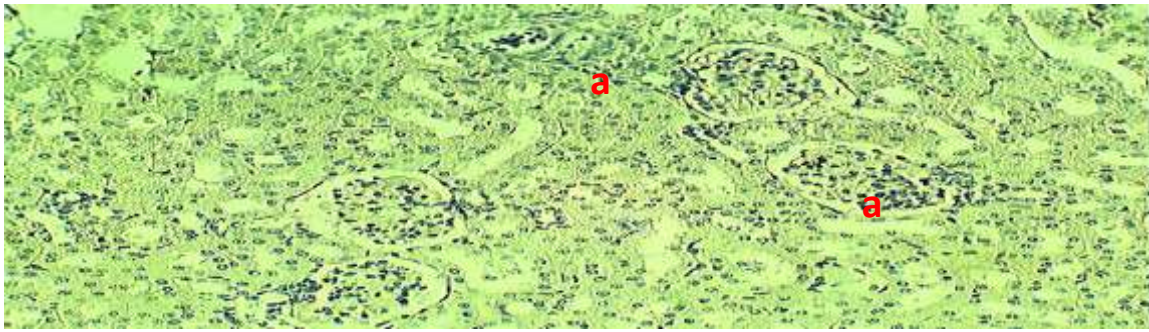

Figure 4. Histopathological section in Kidney tissue of infected mice showed (a) enlarged glomeruli and (b) congestion of the renal B.Vs. (H and E stain, 40X).

Collecting tubules in medulla of kidney with severe degenerative changes of proximal tubules and some of them necrotized, focal MNCs infiltrated the peritubular tissues as demonstrated in figure 5.

Figure 5. Histopathological section in Kidney tissue of infected mice clarified (a) collecting tubules in medulla of kidney (H and E stain, 40X).

Enlargement of glomerular tuft with decreased bowman's spaces, and severe congestion of other glomerular capillary tuft and contained eosinophilic material, the renal tubular epithelium degenerated as showed in figure 6

Figure 6. Histopathological section in Kidney tissue of infected mice clarified (a) renal tubular epithelium degenerated (H and E stain, 20X).

Other section of infected kidney tissue demonstrated renal glomeruli shrinkage due to high cellular immune response caused by *T.* *canis* larvae migration within kidney tissues as showed in figure 7.
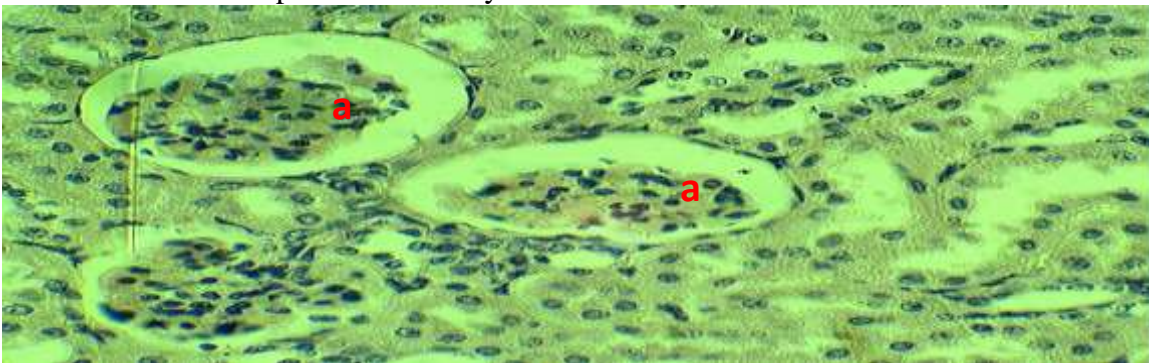

Figure 7. Histopathological section in Kidney tissue of infected mice clarified (a) renal glomeruli shrinkage (H and E stain, 40X).

The presence of inflammatory reactions of mononuclear cells mainly lymphocyte and macrophage in heart and kidney tissues of infected mice with *T. canis* emberyonated eggs was an indication of the moderate to high cellular immune response agents *T. canis* larvae antigens and that deal with Strube *et al.* (28) findings that described a high inflammatory reactions with typical epithelioid granulomata formation around *T. canis* larvae which more frequently occurred in the livers of rats, chickens, guinea pigs at 10 days PI, and in rabbit liver and lung tissues infected with *T. canis* emberunated eggs, likely reflect more effective adaptive immune responses against larval antigens in these species (10). Present study indicated that experimented infected mice suffer from VLM syndrome in heart and kidney tissues with serious complications compared with normal heart and kidney tissue in control group and that deal with (13) results which indicated that clinical spectrum of cardiac involvement in *Toxocara* infections includes myocarditis. While multiple renal glomeruli lesions with presence of eosinophilic accumulation in kidney tissue clarified in this study differ from (2) results that enable detection the migrating of *Toxocara* larvae in kidney tissues. *T. canis* larvae were not found in heart and kidney tissues of infected mice in this study, this deal with other study results and may be due to the larvae continuously migrate (24), or their low bioavailability in these tissues, moreover *T. canis* larvae has been proven to possess efficient strategies to escape the immune attack because larva has ability to coat itself with MUC-120 protein and hide from host immune system (21).

2) **Molecular Detection to *T. canis* in Heart and Kidney tissues:**

Genomic DNA were extracted from heart and kidney tissue successfully, DNA concentration was measured between 34.3-112.2 ng/μl and the purity of DNA ranged between 1.5-1.74 as shown in figure 8. Our results give high *T. canis* larvae DNA concentration compared with (3 and 12) results with 14 ng of *T. canis* larvae DNA.
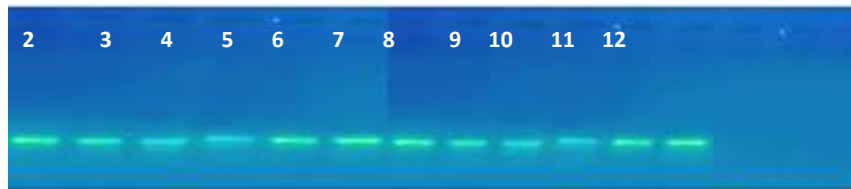
Figure 8. Genomic DNA for all samples (Voltage 60,Gel conce. 0.8%, Time: 1 h).
Molecular identification of virulence larvae *nmuc-1* **gene**

Molecular Identification of *T. canis* larvae based on amplify partial region of virulence gene *nmuc-1*. Our results indicated that two DNA samples extracted from heart tissue and all DNA sample extracted from kidney tissues were belong to *nmuc-1 gene* of *T. canis* larvae stage, figure 9
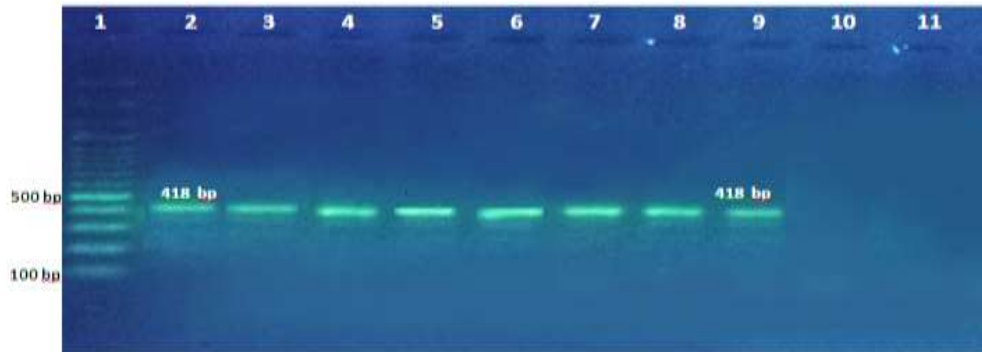
Figure 9. Line 1: Molecular Marker (100bp); lines (2, 3) DNA of *T. canis* **larvae extracted from heart tissue of infected mice; line (4-9) DNA of** *T. canis* **larvae extracted from kidney tissue of infected mice; line (10, 11) negative control (Volt.60, Gel concentration: 1.2% agarose, Time: 1.5 hrs).**

The seropositive rate for human toxocariasis was high in Iraq (9). *T. canis* larvae was the most virulence parasitic pathogen which caused visceral, ocular, nervous, cutaneous or covert larva migrans, however, if embryonated eggs or larvae accidently infected humans, the larvae migrate to the liver, lungs, eyes, or brain, but cannot reach the intestine (27). Larval stage of *T. canis* could release significant amounts of Excretory and Secretory antigens *in vivo* and under *in vitro* culture conditions and the major mucin protein encode by virulence *nmuc-1* gene (23). The presence of *T. canis* larval MUC-120 (mucin) protein highlights the ability of this parasite to resist an attack of the host cellular immune system, allowing survival in host tissues for many years, since the major function of mucin protein was formation of larval surface coat which necessary for larvae invasion and protect from antibody and eosinophil attack during its migration through all body organs tissue (11). Our result is the first study in Iraq that detective *T. canis* larval stage in heart and kidney tissues using virulence *nmuc-1*gene, since it was the only way to detection *T. canis* larvae stage infection in tissue, and confirmed that *T. canis* eggs were hatched on stomach and larvae stage (L2) migrated and recovery at infected mice tissues on first third day post infection. PCR product was sequenced deposited in NCBI with accession numbers LC328969 and at DDBJ and ENA database for the first time in Iraq. Phylogenetic analyses carried out employing (mega 6), depend on Fixation index (F_{ST}), mounted a low genetic variation (0.2) among all comparison isolates and that means minimal genetic diversity among them and that populations are genetically identical as shown in figure 10.
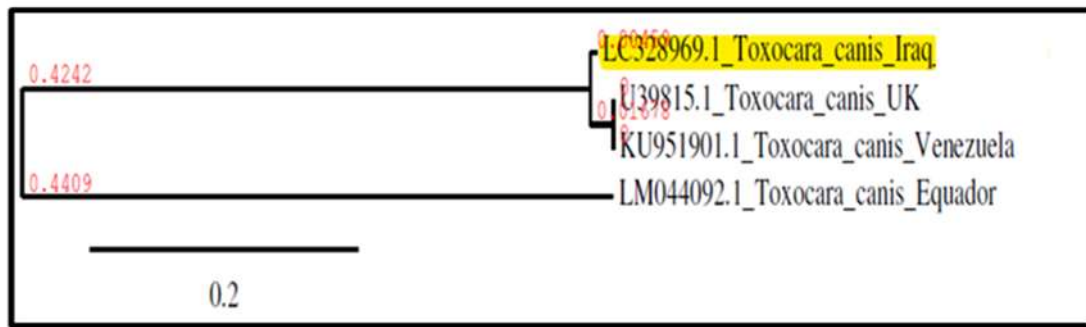
Figure 10. Phylogenetic relationship of aligned sequences of *T. canis* larvae stage

Depending on genetic distance with in phylogenic tree for aligned sequence Iraqi isolate recoded (0.00459) which is more closely to Venezuela and United Kingdom isolates (KU951901 and U39815, respectively) aligned sequence which splits from the same node and recorded (0.01618), than Equador isolate (LM044092) which mounted (0.4409). The result of present study depending on phylogenetic tree analysis indicated that Iraqi *nmuc-1* gene empty from any mutation. The research was able to create simple molecular diagnostic equipment (kit) depending on detection *nmuc-1* virulence gene specific designed primer and PCR technique that enables scientific laboratories in government hospitals to used it for detect and diagnose the infection with *T. canis* parasite (larvae stage) in the patient tissues like human, ruminant and poultry which provides accurate, rapid and inexpensive diagnosis, thus preventing the spread of infection by detecting contaminated meat or products and prevent removed organs surgically. Although a number of scientists used molecular techniques to estimate the genetic parameter and demonstrate evaluate the genetic effects (22 and 30), PCR technique followed by phylogenic tree analysis perfect molecular methods for detection and identification of genetic variants of parasites which agree with Faraj *et al.,* (7). *nmuc-1* gene was virulence gene and can be used as molecular marker to detected VLM syndrome in paratenic host, including human. This study results recommended depend on this gene to ensure if there was an infection with *T. canis* larval stage in tissue of sheep, poultry or cattle local or imported meat or meat products from inside or outside Iraq country and used it for the first time in Iraqi standardization and quality control laboratories as an accurate

microbiological laboratory test used routinely in government hospitals.

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