



Molecular detection of *Toxoplasma gondii* and *Cryptosporidium* spp. of some snakes in AL-Diwaniyah province/Iraq

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Abstract

The Molecular Study, used for the first time in Iraq to detect zoonosis parasites between snakes and humans, included two species of parasites: *Toxoplasma gondii* and *Cryptosporidium* spp., the conventional polymerase chain reaction technique was used to investigate the gene 18S rRNA(382bp) for *Toxoplasma gondii* in the snake tissue of the liver, heart, kidney and brain, 5 samples gave positive result With a total infection rate %16.66 included 3(%60) samples of the *E. jaculus* and one sample(%20) of *P. ventromaculatus* and one sample(%20) of *M. monspessulana* respectively, and the highest percentage of the presence of the gene was recorded in liver of snakes(%80), and the percentage of the lowest in brain %20 The presence of the gene was not recorded in both the heart and the kidney. *Cryptosporidium* parasite where the conventional polymerase chain reaction technique was used as a first step to investigate the SSU rRNA(298bp) gene for the differentiation species of parasites and the DNA sequencer technique, The same gene was used to analyze the evolutionary tree of local isolators that represented *C. parvum* and (MEGA 6) to determine the ratios of convergence between local isolators and International isolators recorded at the International Gene Bank (NCBI). The results of the analysis of the genetic tree of local isolators showed three common ancestors of the genetic groups with the International isolators registered at the International Gene Bank. The samples recorded in NCBI under accession number MG878886, MG878885, MG878884, MG878437, MG878399, MG888048 & MG888047.

Keywords: Snake, Reptile, *Toxoplasma gondii*, *Cryptosporidium* spp., Iraq

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INTRODUCTION

There are about 2,900 species of snakes spread in all countries except the North and South Poles, the Hawaiian Islands, Ireland and New Zealand (Vitt and Caldwell 2013). Reptiles including snakes, are exposed to a variety of pathogens, which may be bacterial, fungal, viral and parasitic, There are many studies have indicated that snakes are the intermediary or definitive hosts for many of the internal parasites, such as round worms, intestinal protozoa such as *Eimeriasp.*, *Isosporasp.*, *Caryosporasp.*, *Tyzzeriasp.*, *Cryptosporidiumsp.* as well as being an intermediate host of *Toxoplasmasp.* (Duszynski and Upton 2009, Klingenberg 2000, Parc 2008, Rataj et al. 2011), as well as blood parasites such as *Plasmodium*, *Haemoproteusmesnili* and *Haemoproteusballi* (Jacobson 2007, Telford 2009), It is also affected by external parasites, most notably ticks such as *Amblyomma sp.* and mites like *Ophionyssusnatricis* (Pietzsch et al. 2006, Rataj et al. 2011).

Toxoplasma gondii, an obligate intracellular protozoan, is responsible for one of the most common zoonotic parasitic diseases in almost all warm blood

vertebrates worldwide, It has been recorded in many hosts including humans, sheep, goats, horses, birds, wolves, snakes, whales, dolphins and seals dogs (Lamourn et al 2001, Nasiri et al. 2016, NZMH 2001).

Cryptosporidium spp. are a complex protozoan parasites that infected a wide variety of vertebrate hosts such as reptiles, birds, amphibians, fish and mammals and cause cryptosporidiosis (Fayer et al. 1997). There are several species classified depending on the species of host, site of infection and the size of the cysts (Xiao et al. 2004). According to our literature review, despite of wide distribution of these snakes in Iraq. There is not comprehensive and adequate published data about *Toxoplasma gondii* and *Cryptosporidium* spp. parasites of these snakes in Iraq. Therefore the current study was conducted to identify *Cryptosporidium* spp. in snakes fecal samples using microscopic and molecular examinations, while *Toxoplasma gondii* using molecular examinations.

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MATERIALS AND METHODS

Sampling

Snakes were collected between March 2017 and March 2018 from five sites of Al-Diwaniyah province (City Center, Afak, East Hamzah, Al-Badair and Nafer districts), then sent to Museum of Natural History at the University of Baghdad for the purpose of diagnosis. Snakes dissection was performed from anus slot towards forefront. The body cavity was opened and the digestive tract was removed, then a small amount of fecal was taken to investigate the *Cryptosporidium* spp. in a direct smear method followed by stain using Zeal Nelson (MZN), positive fecal samples were stored at 4°C before analysis. To investigate parasites *Toxoplasma gondii* whole brain, liver, heart and kidney of snakes were removed and kept at -20 °C until used.

DNA Extraction from Tissues

Liver, kidney, heart and brain were taken to extract the DNA from those tissues and according to the method attached to the test kit processed by the Anatolia Turkish company as follows: cut 10 mg (up to 50 mg) of frozen tissue samples, grind the tissue and transfer to a 1.5 ml microcentrifuge tube for each sample. Added 400 µl of lysis buffer and 20 µl of proteinase K and mix by vortexing, then incubated the mixture for 60 min. at 56°C (lysis step), then added 400 µl of binding buffer and mix by pluse–vortexing for 15s and centrifuge the mixture at 12.500 rpm for 1 minute. Apply the supernatant to the spin column and centrifuge at 12.500 rpm for 2 min (binding steep) and discarded the liquid flow –through into medical waste and placed the spin column back into the same collection tube. Added 500µl of buffer W3 to the spin column (Wash step 1) and centrifuge at 12.500 rpm for 2 min, then discarded the liquid flow –through in to medical waste and place the spin column back in to the same collection tube. Added 500µl of buffer w3 to the spin column (wash step 2) and centrifuge at 12.500 rpm for 2 min. Discarded the collection tube including the liquid flow-through under the spin column in to medical waste and place the spin column in a clean 1.5 ml microcentrifuge tube and wait for 2 min to evaporate ETOH. Added 50µl of buffer EL4 to the spin column and wait for 5 mins (Elution step) and centrifuged at 12.500 rpm for 2 min. The liquid flow –through is the DNA. Then stored at -20°C for later use.

DNA Extraction from Stool

DNA was extracted from snakes stool samples using (Stool Genomic DNA extraction kit) processed by the Korean company Bioneer and extracted according to the company's instructions as follows: Prepared a 1.5 ml microcentrifuge tube and added 20 µl proteinase K, then added 100-200 mg of the stool sample to the tube, buffered to the tube and mix by light vortexing for about 30 second and mix by vortex then incubate for 10 min at 60°C. After 10 mins, centrifuged the tube at 12,000 rpm for 5 mins. Then transferred the supernatant to a new

Table 1. Information regarding the primers used for simultaneous Conventional PCR of *Toxoplasma gondii* and *Cryptosporidium* spp.

Species of parasites	Primers	DNA Sequence	Product length (bp)
<i>Toxoplasma gondii</i>	Forward primer	TTGGATATCCTGCGCTGCTT	382bp
	Reverser primer	CAAGGTGCCATTGCGTTCA	
<i>Cryptosporidium</i> spp.	Forward primer	AGTGACAAGAAATAACAATACAGG	298bp
	Reverser primer	CCTGCTTTAAGCACTCTAATTTTC	

Table 2. Components of reaction mixture Conventional PCR of *Toxoplasma gondii* & *Cryptosporidium* spp.

PCR master mix	Volume
DNA template	5µL
Forward primer(10pmol)	1.5µL
Reverse primer(10pmol)	1.5µL
Master mix	12.5
PCR water	4.5µL
Total	25µL

tube with added 200µl from binding buffer and incubate again for 10 min at 60°C. Added 100 µl isopropanol, lightly vortex for about 5 seconds, then spin down for 10 seconds to down the liquid clinging to the walls and lid of the tube then fit binding column into 2ml collection tube and transfer the liquid into the binding column. Then close lid and centrifuge for 1 min at 8000 rpm. Then transfer the binding column to a new 2ml collection. Added 500µl washing buffer 1(W1) to the column and centrifuged for 1 min at 8000 rpm, then transfer the binding column to a new 2 ml collection tube and added 500µl washing buffer w2 and centrifuge for 1 min at 8000 rpm, then spin down once more at 13,000 rpm for min to completely remove ethanol and transfer the binding column to a 1.5 ml collection tube with 200µl Elution buffer and let stand for min to allow the buffer to permeate the column, then extraction DNA stored in -20 °c for Pending PCR. The purity and concentration of the DNA were measured using Nanodrop spectrophotometer (THERMO.USA).

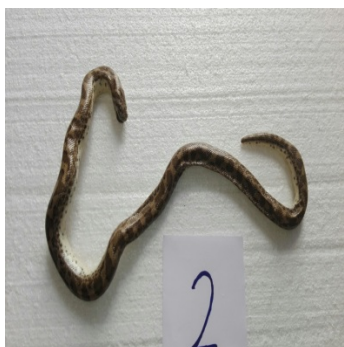
Primer Design

Diagnostic PCR primers were designed to amplify the target gene segment 18s rRNA (382bp) of *Toxoplasma gondii* using Conventional PCR and SSUR RNA(298bp) of *Cryptosporidium* spp. Using Conventional PCR and DNA sequences (Table 1).

Prepare the PCR Master Mix

Prepared PCRmix for parasite *Toxoplasma gondii* and *Cryptosporidium* spp. (and each one separately) using the AccuPower® PreMix kit supplied by the Korean company Bioneer and according to the company's instructions as shown in the Table 2.

After completing the polymerase reaction mixture, the tubes were closed and mixed with vortex for 5 seconds, then tubes transferred to a Thermocycler PCR for thermocycler condition. Amplification reaction of

Fig. 1. *P. ventromaculatus*Fig. 2. *E. jaculus*Fig. 3. *M. monspessulana*

Toxoplasma gondii was conducted with Initial denaturation for 4 min at 94°C followed by 40 cycles of 94°C for 30s (denaturation), annealing at 61°C for 45s, extension at 72°C for 45s and final extension at 72°C for 10 min, while Amplification reaction of *Cryptosporidium* ssp. was conducted with Initial denaturation for 5 min at 94°C followed by 50 cycles of 94°C for 35s (denaturation), annealing at 61°C for 45s, extension at 72°C for 45s and final extension at 72°C for 7 min. Under conditions recommended by the manufactures. The PCR products were analyzed using 1.5% agarose gel electrophoresis.

DNA Sequencing and Phylogenetic Analysis

After polymerase chain reaction, the positive samples of *Cryptosporidium* ssp. (PCR product) were sent to Bioneer in South Korea to define DNA sequence using AB DNA sequencing system to determine the species of *Cryptosporidium* parasite through the

Fig. 4. *N. tessellata*Fig. 5. *D. mesopotamicus*Fig. 6. *S. clifordi*

Phylogenetic tree analysis and NCBI-Genbank-Primer-Blast According to method Morgan et al. (1997).

Statistical Analysis

The results were analysis by Completely Randomized Design (CRD) was adopted as a one-way and two-way laboratory experimental design, as well as a comparison of the averages using a Least Significant Difference (LSD) under probability level $P \leq 0.05$.

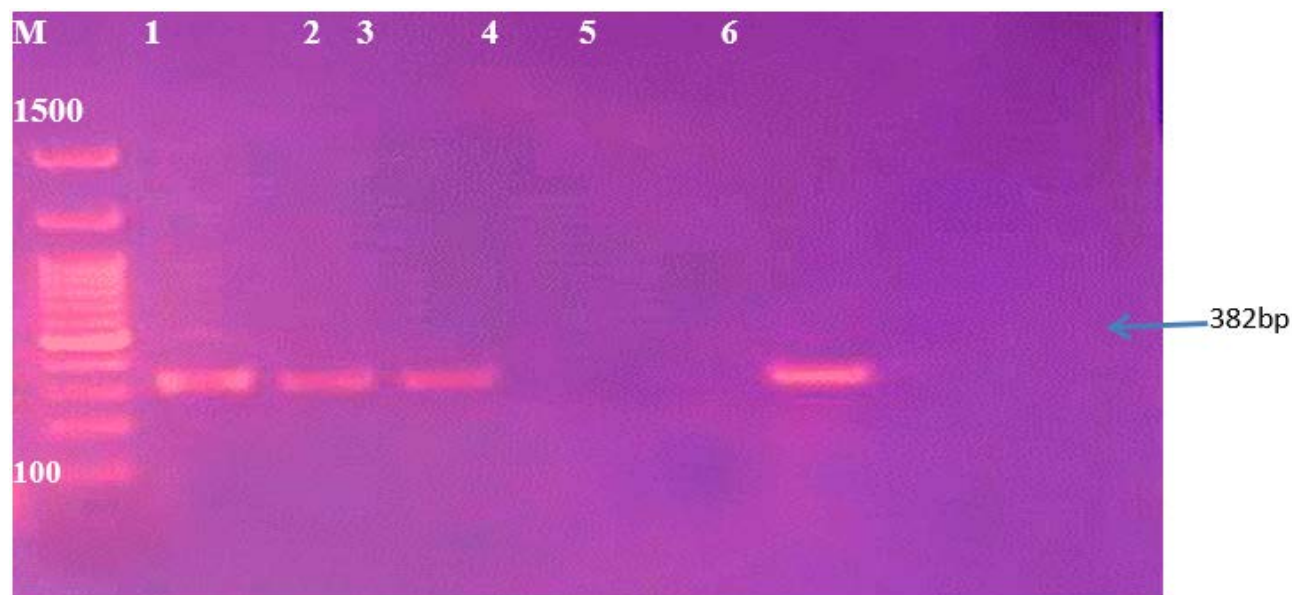
RESULTS AND DISCUSSION

Determination of Positive Samples for *Toxoplasma Gondii* in Snakes Tissue Samples using PCR

There are several different methods to detect protozoa, including microscopic examination, PCR-based techniques, fluorescence in situ hybridization and immunology –based methods. Among them PCR is the most common methods for molecular detection and its

Table 3. Number & percentage of 182 rRNA (382bp) gene to *Toxoplasma gondii* using PCR

Organ infection		Number of samples examined	Number of positive samples of PCR (%)	Liver	Heart	Brain	Kidney
	Species of snakes			N (%)	N (%)	N (%)	N (%)
	<i>Platycephalus ventromaculatus</i>	5	1(20)	1(20)	0(0)	0(0)	0(0)
	<i>Malpolon monspessulana</i>	5	1(20)	1(20)	0(0)	0(0)	0(0)
	<i>Eryx jaculus</i>	5	3(60)	2(40)	1(20)	0(0)	0(0)
	<i>Saplerosophs clifordi</i>	5	0(0)	0(0)	0(0)	0(0)	0(0)
	<i>Natrix tessellate</i>	5	0(0)	0(0)	0(0)	0(0)	0(0)
	<i>Dolichophis mesopotamicus</i>	5	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	30	5(16.66)	4(80)	0(0)	1(20)	0(0)
LSD(≤0.05)		To compare the infection rate between snakes species=N.S To compare the infection rate between infection organ=12.20					
F.calculated:2.08 F.table:2.90 species snakes							
F.calculated:3.64 F.table:3.28 infection organ							

**Fig. 7.** Columns (1-6) represent some positive tissue samples of Conventional polymerase chain reaction where the gene is shown 18srRNA (382bp) of *Toxoplasma gondii*, column M represents a Ladder bearing molecular weight 100-1500

results are more sensitive than microscopic observations in detecting the parasites (Gajadhar and Allen 2004, Ramirez-Castillo et al. 2015). The results of the polymerase chain reaction to detect 182 rRNA (382bp) gene of *Toxoplasma gondii* parasite in liver, heart, brain and kidney tissue samples indicated that the total percentage of the presence of the gene was 16.66% (5 samples) were recorded in only three species of snakes, *E. jaculus*, *P. ventromaculatus* and *M. monspessulana* 60% and 20% respectively. The results did not indicate the presence of the gene in the tissues of *S. clifordi*, *N. tesellata* and *D. mesopotamicus*. It was also noted that the highest percentage of the presence of the gene was in liver samples, 80% (4 samples) and the lowest in the brain as it reached 20% and the study also pointed to the absence of the gene used in the heart and kidney samples (Table 3). The percentage of 16.66% recorded in the current study is a few compared to 80.88% recorded by Nasiri et al. (2016) in the 55 brain snakes out of 68 samples examined using the GRA6 gene. The reason for the lack of positive samples in the present study may be due to the nature of the places where the snakes are located, such as burrows, desert,

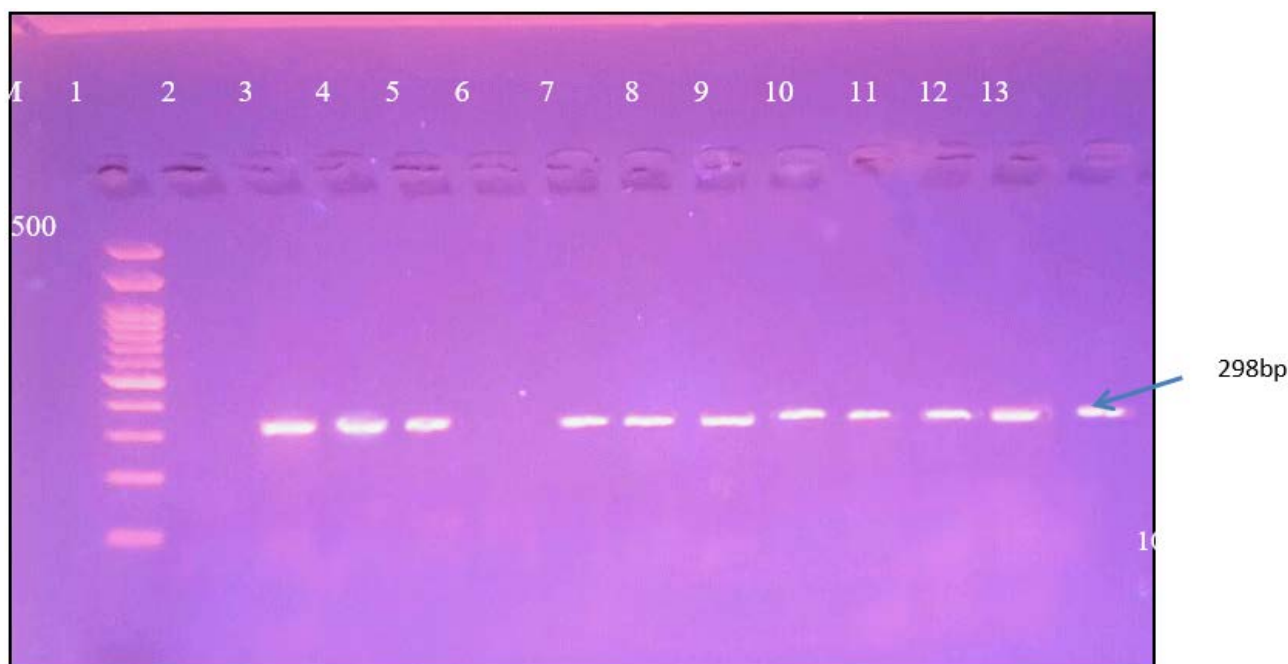
agricultural areas and rivers for aquatic snakes, which there is less presence of the final host represented by cats, in addition to the survival of long periods without food. The high rate of liver infection is attributed to being the first organ responsible for blood filtration and removal foreign substances as well as being rich in nutrients (Gyton and Hull 1995). This parasite was excluded from the DNA sequencer technique for the lack of positive samples of PCR technique and insufficient reaction product to study the gene sequence.

Determination of Positive Samples for *Cryptosporidium* ssp. in Snakes Stool Samples using PCR

The results of the current study showed using the Conventional polymerase chain reaction to detect the SSU rRNA (288bp) of the *Cryptosporidium* parasite in 28 stool samples taken from snakes in the current study, which gave a positive result by direct smear. The total percentage of SSU rRNA was 85.71% (24 samples) (Table 4), and results of the present study showed that there was a decrease in the percentage of the presence of the gene in the samples of aquatic snake compared

Table 4. Number & percentage of SSUrRNA (298bp) gene to *Cryptosporidium* spp using PCR.

Species snakes (n=130)	Number positive using direct smear	Number positive of PCR (%)
<i>P.ventromaculatus</i>	5	5(100)
<i>M.monspessulana</i>	4	4(100)
<i>E.jaculus</i>	3	3(100)
<i>S.clifordi</i>	5	5(100)
<i>N.tessellata</i>	7	3(42.85)
<i>D.mesopotamicus</i>	4	4(100)
Total =130	28	24
LSD(≤ 0.05)=N.S		F.table=4.96 F.calculated=1

**Fig. 8.** Columns (1-13) represent some positive stool samples of conventional polymerase chain reaction where the gene is shown SSU rRNA (298bp) of *Cryptosporidium* spp., column M represents a Ladder bearing molecular weight 100-1500

to the wild species. The presence of the gene SSU rRNA recorded in the current study is higher than the recorded rate by Yimming et al. (2016) in Thailand using the Conventional PCR technique and the same gene of 24.2% with 40 positive samples from 165 stool samples belonging to 8 species of snakes, as well as rate 26% recorded in the *Rhabdophis tigrinus* by Kuroki et al. (2008) in Japan. Differences in the ratios recorded in the current studies and global studies may be due to the different species of examined snakes, their numbers and laboratory conditions uncontrolled.

Matching *Cryptosporidium* Parasite Sequences in this Study with Sequences of Parasite Species in NCBI Genbank and the Same Gene

Multiple sequence alignment analysis results showed for the products PCR reaction based on the SSU rRNA gene of *Cryptosporidium* parasites. After comparing the *Cryptosporidium* sequences isolated in the current study with sequences of *C. parvum* parasites recorded in the NCBI and the same gene, only 7 samples were identical with the genus *C. parvum* recorded globally and with a percentage of compatibility

ranging from 100-99%. The small subunit ribosomal (18S rRNA) encoding gene has been used to provide a lot of information about this gene at the National Biotechnology Information Center on the one hand and for the other Own a recipe Stability and variability among species (Xiao et al. 1999b).

The registration of *C. parvum* in Iraqi snakes either in direct smear or molecular methods are the first recorded in Iraq as a zoonosis parasite between snakes and humans, Local studies have not indicated its registration previously. The current study has agreed with some international studies that indicated the possibility infection of various species of snakes *C. parvum* one of these studies Yimming et al. (2016) in Thailand using Nested PCR and DNA sequencer, and Xiao et al. (2004) in Czech Republic, Republic of Ghana and Australia in his study of a group of reptiles including snakes, while it differed with Graczyk et al. (1996) which indicated that *C. parvum* was not transmitted to reptiles, fish or amphibians.

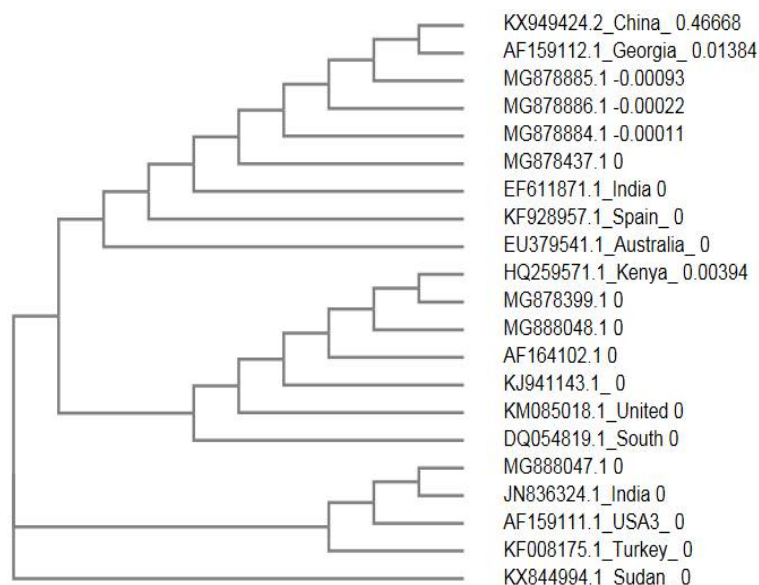


Fig. 9. Analysis of the genetic tree using the MEGA 6 program where the UPGMA tree test was used. The results show a common genetic relationship in the local parasite samples taken from the Iraqi snake stools with the *C. parvum* parasite recorded in NCBI-Genbank

Phylogenetic Tree Analysis of *Cryptosporidium Parvum* Isolated from Iraqi Snakes Stool

Results of the tree analysis of local isolators based on SSU rRNA gene showed three common predecessors of the genetic groups (**Fig. 9**), the first genotype where the local isolators of *C. parvum* parasites with accession numbers MG878885.1, MG878886.1, MG878884.1 and MG878437.1 were shown to have a genetically close relationship with some of the isolators registered at the International Gene Bank such as China, Georgia, Indian, Spain and Australian isolators recorded by Zhang et al. (2016), Xiao et al. (1999), Paul et al. (2007), Fernandez-Alvarez et al. (2013) and Jex et al. (2008) respectively, and local isolator with accession number MG878885.1 was the closest isolators to the Chinese and Georgian isolators and far from the rest of the isolates.

The second genotype was associated local parasite isolators, which bore accession number MG878399.1 and MG888048.1 genetically linked to Kenyan isolates and isolates of the United Kingdom and South Korea registered in the International Gene Bank by, Kangethe et al. (2010), Hadfield et al. (2015) and Park et al. (2006) respectively. Local isolator MG878399.1 was the closest isolator of Kenyan. While the third group showed that the local isolator of the parasite with the accession number MG888047.1 has a genetic link close to the isolators of India, United Kingdom, United States, Turkey and Sudanese registered in the gene bank from by Maurya et al. (2011), Xioa et al.(1999), Ciloglu et al.(2013) and Taha et al.(2016) respectively.

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