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RESEARCH ARTICLE

Investigation of Toxoplasmosis in Human, Camels and Goats by Using Molecular Methods

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ABSTRACT

The current study included the detection of *Toxoplasma gondii* in camels, goats from slaughter house in Al-Qadisiyah province, aborted ewe from owner who attended to the veterinary hospital in Al Diwanyah and also from aborted women from Maternity and Childhood Teaching Hospital to point to the rate of infection in the human and to study animals and also the organs can be found inanimals. A total of 200 samples distributed as a males and females of camels and goats, and from aborted women , the samples were divided into, (70) sample from camels, from (liver, meat and uterus), 70 samples of goats' meat, uterus, placenta and fetus and 60 samples were collected randomly from aborted women who received to the Maternity and Childhood Teaching Hospital. The first method of diagnosis was an impression method for all samples. The result of camls shows that was 48/ 70 positive (68.57%), while the result of goats were 45/70 (64.28%) and the aborted women show 20/60 positive (33.33%) , there was a statistically significant among the species in p <0.05. to suspected *toxoplasma gondii* bradizoite.The present study include molecular method to detected small subunit ribosomal RNA (ssRNA) using Nested Polymerase Chain Reaction (nPCR) ,the results of the nPCR for ssRNA gene showed that 18/32 (54.54%) were positive in the camels , while the result of the goats were 17/33 (51.51%) were positive, the rate of toxoplasmosis in the aborted women was Positive 60%. There is a statistically significant between species in p<0.05.

Keyword: Toxoplasmosis,NPCR, Camel,goat, human, Diwaniyah, Iraq.

INTRODUCTION

Toxoplasmosis is a classic zoonosis, A wide range of vertebrate animals serve as hosts and while human infections are common, serious complications occur primarily inimmune compromised hosts, Human infections are caused by accidental ingestion of oocytes, shed into the environment by cats, or tissue cysts contained in undercooked meat, Infections in healthy adults are generally benign,although toxoplasma retinitis is frequently a cause of serious eye disease in otherwise healthy adults (Gilbert et al., 1999). More profound disease occurs in immune compromised hosts or as the result of congenital infections (McLeod et al.,2000). Toxoplasmosis intracellular parasite that has a two stage asexual cycle in warm-blooded animals and a sexual cycle in felidae, the parasite comprises three clonal lineages (I, II and in the main, with type II and III being associated with disease in animals while type II is the predominant form identified in human disease (Howe and Sibley, 1995, Khan et al., 2006). In the asexual cycle, the two developmental stages are the rapidly multiplying tachyzoite and the slowly multiplying bradyzoite, in acute infection, tachyzoites actively penetrate host cells where they multiply causing the cell to rupture and release organisms locally and into the bloodstream As the host develops immunity, the parasite retains its overall size and shape but transforms into the bradyzoite stage and multiplies more slowly within tissue-cysts to establish a persistent infection, These microscopic tissue cysts are present most frequently in brain and skeletal muscle and represent the quiescent stage of the parasite within the host, Viable tissue cysts within muscle (meat) are a significant source of human infection, In animals that succumb to acute infection tachyzoites may be demonstrated in ascitic fluid or in lung impression smears as well as in tissue sections of the liver and other affected organs (Luft et al.,1993).

MATERIALS AND METHODS

Primers

The Nested PCR primers for detection small subunit ribosomal RNA gene in *Toxoplasma gondii* were design by pervious study (Vitale et al.,2008).These primers were provided from Macrogen company, Korea as following table:1

Samples collection

The study was carried out during the beginning of November 2017 until thend of febrewary 2018 in AL-Qadisya provenance, total 200 samples were examined in the current study divided as 70 samples of different camel organs (liver, meat, utrues) in the slaughter house, 70 samples of goat also taken from slaughter house (uterus,meat, Placenta andfetus).Also the present study include 60 human samples from aborted women (placenta and fetus) who attended to the Maternity and Childhood Teaching Hospital. The samples were collected randomly and using two clean Container containing 1st one containing normal saline and kept cool and transferred to the laboratory to make the imppsresion method and the 2nd one then putin deepfreeze under for DNA extraction as a target for PCR amplification.

Microscopic Examination

(by stamp method or immersion method) Stamp the tissue on the glass slid and left in air to dray and methanol alcohol was added for (3-5)minute then was stained with Giemsa stain for 30 mints and was washed by tab water then left to dry, after drying they were examined under the oil immersion lens of light microscope (Zafar *etal*., 2006).

Tissue Genomic DNA Extraction

Tissue Genomic DNA from tissue samples were extracted by using DNA extraction kit (Tissue DNA protocol) Geneaid. USA and the extraction was done according to company instructions as following steps:

1. Weight of 100mg Human tissue samples was transferred to sterile 1.5ml microcentrifugetube.
2. Volume of 200µl GST 20µl and proteinase K were added and mixed by vortex. And incubated at 60℃for 1 hour.
3. Volume of 200µl of GSB was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70℃ for 15 minutes, and inverted every 3 minutes through incubationperiods.
4. Volume of 200µl absolute ethanol were added to lysate and immediately mixed by shakingvigorously.
5. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tubes containing the flow-through were discarded and placed the column in a new 2 ml collectiontube.
6. Volume of 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collectiontube.
7. 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collectiontube.
8. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.
9. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre-heated elution buffer were added to the center of the columnmatrix.
10. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purifiedDNA.

Nested Polymerase chain reaction (nPCR)

The nested PCR technique was performed for detection *Toxoplasma gondii* based small subunit ribosomal RNA gene from all tissue samples of all specise. This method was carried out according to method described by (Vitale *et al*., 2008) as following steps:

Genomic DNA estimation

The extracted genomic DNA was by using Nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank thesystem.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of DNA was added tomeasurement.

3-2-6- Primary PCR master mix preparation

Primary PCR master mix was prepared by using (Maxime PCR PreMix Kit) and this master mix done according to company instructions as following table:2 After that, these PCR master mix component that mentioned in table above placed in standard PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (T100 Thermal cycler. BioRadUSA).

PCR Thermocycler Conditions

PCR thermocycler conditions by using convential PCR thermocycler system as following table:3

Secondary PCR master mix preparation

Secondary PCR master mix was prepared by using (Maxime PCR PreMix Kit) and this master mix done according to company instructions as following table:4 After that, these PCR master mix component that mentioned in table above placed in standard AccuPower® PCR PreMix Kitthat containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye).Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (T100 Thermal cycler. BioRad USA).

PCR Thermocycler Conditions

PCR thermocycler conditions by using convential PCR thermocycler system as following table:5

PCR product analysis

1. The Nested PCR products was analyzed by agarose gel electrophoresis following steps: 1-A percent of 1% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool50°C.
2. Volume of 3µL of ethidium bromide stain were added into agarose gelsolution.
3. Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in onewell.
4. The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for1hour.
5. Nested PCR products were visualized by using UVTransilluminator.

RESULTS AND DISCUSSION

Detection of *toxoplasma gondii* bradyzoite using impression method

Detection of Toxoplasmosis incamel

Incidence of suspected bradyzoites Toxoplasmosis according toorgan

The present study was used impression method for detect suspected toxoplasma btadyzoites in different organs of camel, 70 samples were examined (9 liver, 25 meat and 36 uterus). The results shows the percent of the infection in general 48/70 (68.57%), while the rate of the infection according to the organs were 3/9 (33.33%), 12/25 (48%) and 33/36 (91.66%) in the liver, meat and uterus respectively table (1), figure (6). The preset study divided the camel samples based on gender into 28 male and 42 female, the infectious rate of toxoplasmosis according to the gender based on impression method were 9/28 (68.57%) and 39/42(92.85%) in male and female respectively table(7). In the study found the infectious rate of toxoplasmosis was 68.57%,this result agree with other study using the LAT as a serological test in the Sudan 67% (Elamin etal.,1992), also another prevalence study for *Toxoplasma gondii* seropositivity detection in Sudan using the LAT was (61.7%)(Manal ,2003)

Detection of Toxoplasmosis ingoat

Incidence of suspected bradyzoites Toxoplasmosis in the goat according to theorgans

The current study use impression method to detected suspected toxoplasma bradyzoite in the uterus, meat, placenta and fetus samples from slaughter house. The result of the present study was 45/70 (64.28%) was positive result in the method above., while according to the organs were 46.66 %, 73.33%, 73.33% and 64% in the uterus ,placenta , fetus and meat respectivlly table(8) figure(2). The preset study divided the goat samples based on gender into 25 male and 45 female, the infectious rate of toxoplasmosis according to the gender based on impression method were 16/25 (64%) and 29/45(64.44%) in male and female respectively table(9).The present study found 64.28% in the goat, On the other hand the result of another study which were nearlyto the result of the present study in Bangladesh reported 61% by used latex method (Rahman etal.,2014) , in Egypt reported 59.4% by used indirect hemaaglutination assay, (Barakat etal.,2009).

Detection of Toxoplasmosis in human

Incidence of bradyzoites Toxoplasmosis according to organ

The current study used the impression method to detected toxopasmosis bradyzoite in the placenta and fetus from the same aborted woman who attended to the maternity and childhood hospital in Al Diawanyia province. A total number of placenta and fetus samples were (60 each sample to the single patient) samples exam by impression method, and the result of this study found 20 /60 (33.33 %) positive That means the placenta source of transmission of infection to the fetus. Table (10) figure (3). The present study found 33.33% in the human, In other hand by ues ELISA test the seroprevalence in women was 50% in USA (Stagno, 1980), 54% in Kenya (Griffin and Williams, 1983), 7.5% in Scotland (Jackson and Hutchinson, 1987).

Detection of *Toxoplasma gondii* using Molecular methods.

DNA extraction

The DNA of positive samples to impression method which are (95) was extracted and purificated by using genome DNA purification kit. The results were detected by nanodrop and the result were exelant.

Nested polymerase chain reaction nPCR to detect small sub unit ribosomal RNA gene.

The results of PCR amplification which was performed on the DNA extracted of small subunit ribosomial RNAgene of *Toxopalasma gondii*, the samples were taken from 95 samples which were positive result with impression method as (32,33and 30) camel,goat and human respectively the studied isolates confirmed by electrophoresis analysis. By this analysis the strands of DNA which are resulted from the successful binding between specific primers and extracted DNA of isolates, These successful binding appear as single band together with the 313 pb band product size.The result of nPCR in present study of camel was 18/32 (54.45%) from positive samples to impression method, while the result of goat in the same method was 17/33% figure (4). The electrophoresis also used to estimate DNA weight depending on DNA marker (2000 bp DNA ladder) and the result of this estimation revealed that the amplified DNA figure(5). Out of 95 were taken from impression methodpositive cases, and examined by nested PCR found (53) positive result with a percent (55.20%).In human the number of samples was 18/30 from positive impression method Table (8).

The correct study found 54.54% in camel, other study use LAT of Al-Qadisiya province reported 16.34%(Al-Hindawe,2006) , Hilali *etal.* (1998) detect aprevelance of 17.4%. The differences among percentages that, recorded in the current study and other studies may be due to method of diagnosis or the time of collected samples.The correct study found 51.51% in goat, this result compare with other result use the blood sample for an indirect antibody test in the slaughterhouse in Iran (Sharif *etal*., 2007) which reported 30%.The present study found 60% in the human, while the result of Al- Kalaby, (2008) who recorded that 83.3% of tested samples from Iraq women was positive by PCR technique using *B1* gene, on the other hand the results of Okay *et al.,* (2009) who reported that 17.65%, the result of present study agreed with the results of Al-Addlan, (2007) who reported that 63.49%

CONCLUSIONS

1. This study determine the proportion of *Toxoplasma gondi* parasite in the organs of slaughter animals and in humans by using the different technique , the study shown that the presence of parasite in a high rate in the camel
2. There is a variation in the incidence of infection among different animals andhuman
3. There is a variations in the incidence of the infection of toxoplasma gondi parasite between different organs of the camel depending on the stage of infection and the importance of the organ relative to theparasite
4. The uterus is considered the best organs as a target of the toxoplasma gondi when compared to the othersorgans.
5. Meat of slaughter animals is an important source of toxoplasmosis and a threat to consumer health.

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Table 1. PCR primers RNA gene in *Toxoplasma gondii*

Primer	Sequence		Amplicon
18SrRNA gene First round PCR Primers	F	TGCGGAAGGATCATTCACACG	530bp
	R	CCGTTACTAAGGGAATCATAGTT	
18SrRNA gene Second round Nested PCR Primers	F	GATTTGCATTCAAGAAGCTGATAGTAT	313bp
	R	AGTTAGGAAGCAATCTGAAAGCACATC	
DNA sequence Toxo-SAG3primers	F	ATGCAGCTGTGGCGGCGCAG	1158bp
	R	TTAGGCAGCCACATGCACAAG	

Table.2. Maxime PCR PreMix Kit

PCR Master mix	Volume
DNA template	5µL
18SrRNA primary Forward primer (10pmol)	1µL
18SrRNA primary Reverse primer (10pmol)	1µL
PCR water	13µL
Total volume	20µL

Table.3. thermocycler system

PCR step	Temp.	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	
Annealing	58C	30sec	40 cycle
Extension	72C	30sec	
Final extension	72C	5min	1
Hold	4C	Forever	-

Table.4. Secondary PCR master mix

Nested PCR Master mix	Volume
PCR product	2µL
18SrRNA secondaryForward primer (10pmol)	1µL
18SrRNA secondary Reverse primer (10pmol)	1µL
PCR water	16 µL
Total volume	20µL

Table.5. thermocycler system

PCR step	Temp.	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	
Annealing	58C	30sec	40 cycle
Extension	72C	30sec	
Final extension	72C	5min	1
Hold	4C	Forever	-

Table 6. the percentage according to organs suspected of bradyzoites of toxoplasmosis in camel

Organ	No.	(+ve)	%
Liver	9	3	33.33^{B}
Meat	25	12	48^{B}
Uterus	36	33	91.66^{A}
Total	70	48	68.57

Similar litter represent no statistically significant at $p < 0.05$
Different letter represent statistically significant at $p < 0.05$

Table 7. The percentage according to gender suspected to bradyzoites of toxoplasmosis in camel

Sex	N0.	Number(+ve)	%
Male	28	9	32.14^{B}
Female	42	39	92.85^{A}
Total	70	48	68.57

Different letter represent statistically significant at $p < 0.05$

Table 8. Show the percentage according to organs suspected to bradyzoites to toxoplasma in goat

Organ	N0	(+ve)	%
Uterus	15	7	46.66A
Placenta	15	11	73.33B
Fetus	15	11	73.33B
Meat	25	16	64A
Total	70	45	64.28

Similar litter represent no statistically significant at $p < 0.05$
Different letter represent statistically significant at $p < 0.05$

Table 9. the percentage according to gender suspected to bradyzoites of toxoplasmosis in goat

Sex	N0.	Number(+ve)	%
Male	25	16	64^{B}
Female	45	29	64.44^{A}
Total	70	45	64.28

Different letter represent statistically significant at $p < 0.05$

Table10. the percentage of toxoplasmosis according to organs suspected to bradyzoites to toxoplasmosis in human

Organ	N0	(+ve)	%
Placenta	60	20	33.33
Fetus	60	20	33.33
Total	60	20	33.3

Table 11 Compression of the result of nPCR according to the different species

Species	nPCR	(+ ve)	%
Camel	32	18	54.54^{A}
Goat	33	17	$51.51\%^{A}$
Human	30	18	$60\%^{A}$
Total	95	53	55.20%

Similar litter represents no significant difference at $p < 0.05$

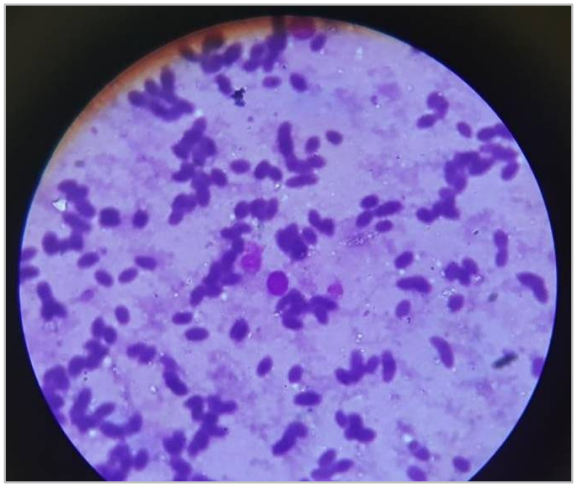

Figure 1.the bradyzoite in camel using Impression method, staining with Gemisa (X100).

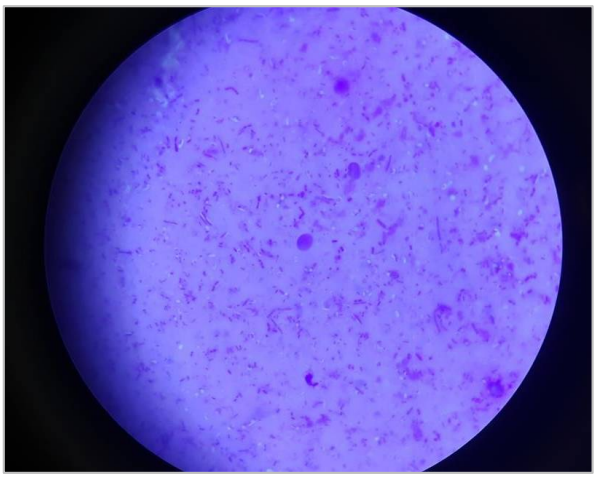

Figure 2. The bradyzoite in goat using impression Method and staining with Gemisa (X100).

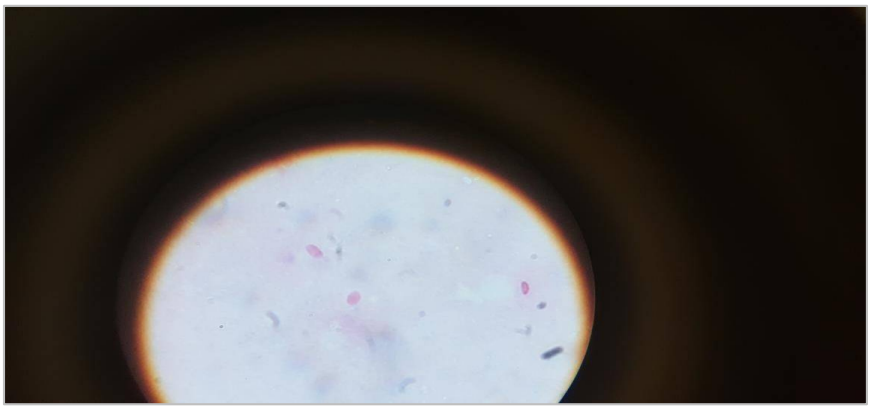

Figure 3. the bradyzoite in human using impression method and staining with Gemisa stain (X100)

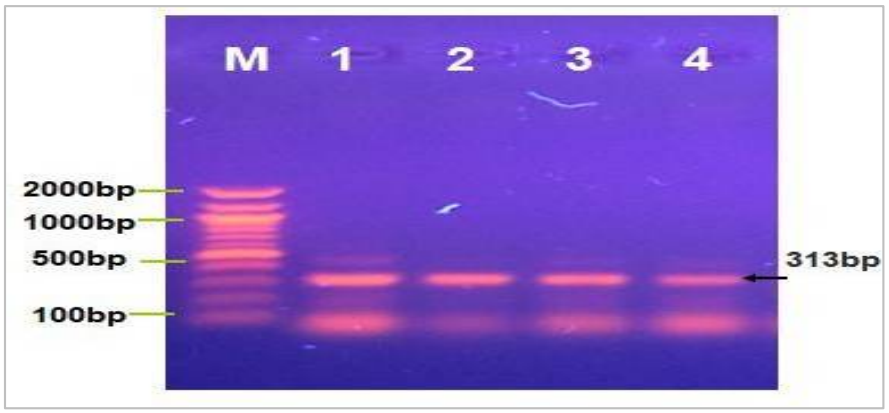

Figure 4. Agarose gel electrophoresis image that show the NestedPCR product analysis of small subunit ribosomal RNA gene in *Toxoplasma gondii*. Where Marker ladder (2000-100bp), (1-4) some positive *Toxoplasma gondii* from camel samples at 313bp PCR product size

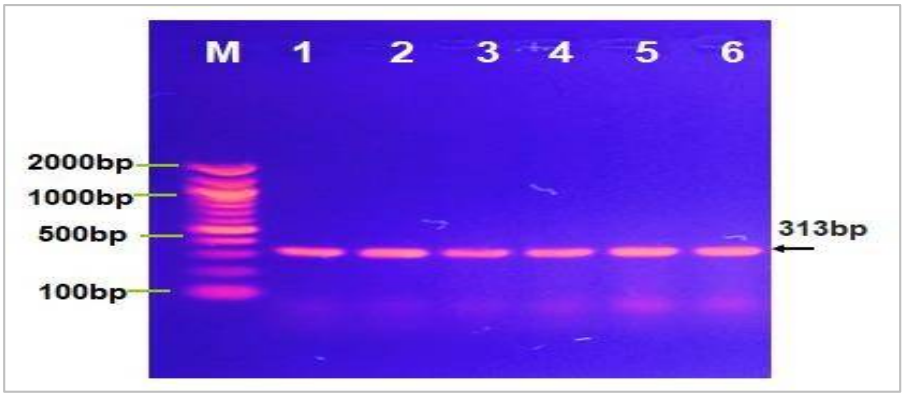

Figure 5. Agarose gel electrophoresis image that show the Nested PCR product analysis of small subunit ribosomal RNA gene in *Toxoplasma gondii.* Where Marker ladder (2000-100bp), (1-6) some positive *Toxoplasma gondii* from goat samples at 313bp PCR product size