Molecular study of the giardia parasite in the beauty of the province of Diwaniyah

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ABSTRACT

Because of little studies which related with camels in Iraq, our study aimed to description of Giardia lamblia in it, in parasitology laboratory of Life Sciences in Qadissiya University.The current study included a random examination of 100 fecal samples of camels from slaughterhouses and Bedouin areas in Diwaniyah Governorate. Some camels suffered from diarrhea and other asymptomatic animals from October 2019 to the end of April 2020, and their ages ranged from random examination. The infection with giardiasis (40 samples, 20%) was by direct smear, flotation method, and by using light microscopy to detect parasite phases and cysts. The incidence of giardia parasites was 19.3%.The prevalence rate according to the age of the camel, Giardia infection was higher at age less than 44.4%, while the rate decreased at age more than 17.5%. According to the most popular study of giardia infection, the highest range is 28.1% in January and the lowest range is 3.3% in December. Statistical analysis of results showed there is significant difference in both parasite infections among the age range and months of study.Regarding the prevalence of parasites, 100 faecal samples were examined using polymerase chain reaction (PCR) technique. The prevalence of giardia (39%) in Diwaniyah.This study showed that camels are the source of infection with the studied parasites, which are considered parasites of animal origin.

Keywords: Giardia, parasite, Molecular study

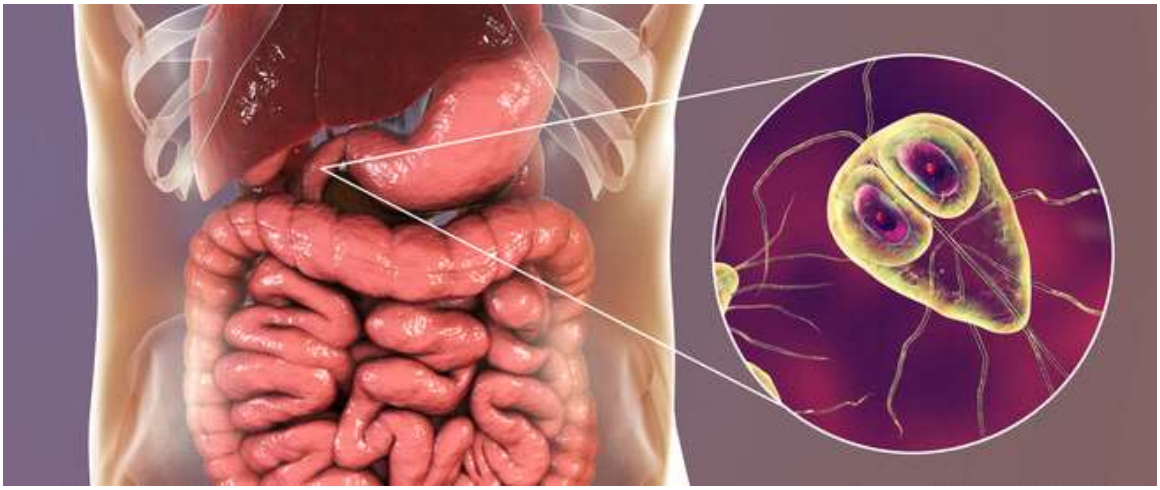
Introduction

Giardia are enteropathogenic parasites of humans and animals, producing asymptomatic to severe intestinal infections (Didier, 2005; Xiao and Fayer,2008).

It is an intestinal parasite spread throughout the world, between humans and animals. This parasite has

neither mitochondrion nor even the Golgi apparatus, and is therefore considered one of the primitive forms of eukaryotes.

Giardia disease can cause dehydration and hinder your body from functioning.



Giardia lamblia (Syn; *Giardia duodenalis, Giardia intestinalis*) is the causative agent of giardiasis, a gastrointestinal infection of humans, companion animals, livestock and wild life, signs of a *Giardia* infection vary from asymptomatic to profuse diarrhea as well as chronic disease (Feng and Xiao, 2011). *Giardia lamblia* has a simple life cycle comprising rapidly multiplying, non-invasive trophozoite on the mucosal surface of the intestine, and production of environmentally resist at cysts that are shed with host feces (Adam, 2001).
Diagnosis involves testing for *Giardia* trophozoites and cysts in direct unstained fecal smears to look for motile trophozoites or using Lugol's iodine to help distinguish the cysts and trophozoites (Barr and Bowman, 2006).
The common methods of detection of *Giardia lamblia* in feces were enzyme-linked immunosorbent assay (ELISA), microscopy and/or real-time PCR (Van den Bossche *et al.,* 2015).

Aim of the study
Giardia disease is a zoonotic disease, and no studies have been conducted on giardia in camels in Iraq, which may be a source of infection.

- *Diagnosis the causative agent (protozoa) of diarrhea in camels by microscopic examination.*
- *Molecular detection of protozoa species by PCR (conventional - PCR).*
- *The sequence of detection of giardia andersoni in camels first in Iraq.*

Materials and Methods
Materials
Chemical and Biological materials:
The chemical and biological materials that were used in this study are listed in Table (1).

Table (1) Chemical and biological materials used in study.

Origin	Name of chemical and biological materials	No.
Germany	Acid Alcohol stain	1.
Germany	Basic Fuchsin stain	2.
England	Crystal iodide	3.
Iraq	Ethyl Alcohol	4.
U.K	Formaldehyde 40%	5.
India	Normal saline	6.
England	Oil immersion	7.
Germany	Phenol Methelyne Blue stain	8.
Evans / Liver pod	Potassium iodide	9.
England	Xylol	10.
England	Zinc sulphate	11.

Equipments and Instruments

Table (2) The equipment and instruments that were used in this study.

Company	Equipment & instrument	No.
Sturdy (Taiwan)	Autoclave	1
Jorden	Blastic tubes 10ml	2
China	Cover slides	3
Jorden	Container 50ml	4
China	Cotton	5
Samsung/ china	Digital camera	6

Sterile EO. / China	Disposable syringe 10 ml, 5ml and 3ml	7
China	Disposable Gloves (large, meddle)	8
Bioneer/ Korea	Eppendorf tubes	9
Bioneer/ Korea	Exispin centrifuge	10
Shandod Scientific/ UK	Gel electrophoresis	11
Eppendorf /Germany	High Speed Cold centrifuge	12
Mammert/Germany	Incubator	13
China	Lint	14
Olympus / Japan	Microscope	15
China	Microscope slides	16
CYAN/ Belgium	Micropipettes 0.5-10,5-50, 100-1000µl	17
Mammert/Germany	Oven	18
Concord /Lebanon	Refrigerator	19
Sartorius/Germany	Sensitive Balance	20
Superestar/ India	Sterile test tube	21
China	Sticks	22
India	Test tubes	23
MyGene/ Korea	Thermocycler PCR	24
ATTA/ Korea	UV Transilluminator	25
CYAN/ Belgium	Vortex	26
Mammert/Germany	Water Bath	27

Table (3): The kits used in this study with their companies and countries of origin:

Country	**Company**	**Kit**	**No.**
Korea	**Bioneer**	**AccuPrep® stool DNA Extraction Kit**	**1**
		Proteinase K	
		Stool Lysis buffer (SL)	
		Binding buffer (ST)	
		Washing buffer 1 (W1)	
		Washing buffer 2 (W2)	
		Elution buffer (E)	
		GD column	
		Collection tube 2ml	
Korea	**Bioneer**	**AccuPower® PCR PreMix Kit**	**2**

		Taq DNA polymerase	
		dNTPs (dATP, dCTP, dGTP, dTTP)	
		Tris-HCl pH 9.0, KCl, $MgCl_2$	
		Stabilizer and Tracking dye	

Primers

Three PCR primers were designed in this study for detecting *G. lamblia*, *Cryptosporidium* spp. and *Cryptosporidium andersoni* based subunit ribosomal rRNA gene by using NCBI-Genbank (M54878.1, AF112573.1, and AY881992.1 respectively) and primer3 plus design online. Then these primers was provided from Bioneer company, Korea as following table (Table 3-4)

Table (4):Primers used in this study with their Sequence and PCR Size:

Amplicon	Molecular weight	Sequence		Primer
16s-like RNA	**567bp**	**GTTGAAACGCCCGTAGTTGG**	**F**	***Giardia lamblia***
		GTTGTCGCAATGGAGCAGAC	**R**	

Chemicals :

Table (5):The chemicals of PCR that were used in this study.

Company and Origin	Chemical	No.
BDH (England)	Absolute Ethanol	1
BioBasic (Canada)	Agarose	2
BioBasic (Canada)	Ehidium Bromide	3
Bioneer (Korea)	Loading dye	4
Bioneer (Korea)	PCR water	5
BioBasic (Canada)	Proteinase k	6
BioBasic (Canada)	TBE buffer	7

Solutions for detection of *Giardia lambelia*

1-Formal saline (10 %)

Prepared by mixed 100 ml of formaldehyde (40 %) were added to 900 ml of normal saline (0.85 %) (Levine, 1961).

2-Lougal iodine (1 %)

One gram of crystal iodide was dissolved in 100 ml of distal water and mixed thoroughly, then 2 grams of potassium iodide were added to the solution and mixed until dissolved completely (Coles, 1986).

Methods

Collection of stool samples

100 stool samples were collected from camels. Some of these camels suffered from diarrhea and some asymptomatic camels in domestic slaughter and other areas in Diwaniyah Governorate during the period from October - 2019 until the end of April - 2020, except for February and from the age of 1. From one year to more than a year.

These samples were collected in sterile plastic containers and stored in large packages containing ice bags, and then transferred to the Parasitology Laboratory in Life Sciences / University of Al-Qadisiyah for examination.

Microscopic examination:

- **The direct smear method by Lougal Iodine:**

  According to Coles (1986) the method was done was following:

  - A drop of lougal iodine solution was placed on a glass slide.
  - Small amount (about 1 gm) was put on lougal iodine drop and mixed thoroughly using wooden stick.
  - Cover slide was applied with forceps or fingers.
  - Examined of slide under (40 x) and (100 x) powers with oil emersion lens.

- **loatation method**

  Zinc sulfate solution consider the best solution usage for floatation method , according to mixed the feces with high qualitative weight as saturated zinc sulfate solution which prepared from dissolve 33 gm of saturated zinc sulfate solution in 100 millilitters of distaled water . the examination by floatation method as following steps: (Coles,1986)

  - Mixed 3 gm of feces with distaled water weller then filtered through two layers of lint in picker .
  - Put the solution in test tubes and entered in centrifuge at speed 3000 rpm for two minute, the sediment was taken and discard the floater .
  - Diluted the sediment with distaled water and repeated centrifuge for 3 times at speed 3000 rpm for two minute.
  - Added saturated zinc sulfate solution to sediment then centrifuged at speed 3000 rpm for two minute .
  - Taken the foam in upper by pipette then put on the slide for microscopic examination .

Polymerase chain reaction (PCR)

The conventional PCR technique was performed to detection of *G. lamblia* and *Cryptosporidium* spp. based 18 subunit ribosomal RNA gene(18srRNA) from camel stool samples. This method was carried out according to method described by (Verweij *et al*., 2004) as in the following steps:

A- Genomic DNA Extraction

Genomic DNA from camel feces samples were extracted by using AccuPrep® stool DNA Extraction Kit , Bioneer. Korea, and done according to company instructions as in the following steps:

- A 200 mg of the stool sample was transferred to sterile 1.5ml microcentrifuge tube, and then 20µl of proteinase K and 400 µl Stool lysis buffer (SL) were added mixed by vortex. And incubated at 60℃ for 10 minutes.
- After 10 mins, the tube placed in centrifuge at 12,000rpm for 5 minutes.
- The supernatant was transferred in to a new tube and 200µl Binding buffer was added to each tubes.
- The tubes were Incubated again for 10 min at 60℃.
- 100 µl isopropanol was added and the samples mixed by 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.
- 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 8000rpm for 5 minutes, and the 2 ml collection tube containing the flow-through were discarded and placed the column in a new 2 ml collection tube.
- 500µl 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 collection tube.
- 500µl W2 Buffer (ethanol) was added to each column. Then centrifuged at 8000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.
- All the tubes were centrifuged again for 1 minutes at 12000 rpm to dry the column matrix.
- 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 column matrix.
- 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 purified DNA.

B- Genomic DNA Profile

The extracted DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance 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 the system.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of DNA sample was added to measurement.

C- PCR reaction solution

PCR master mix was prepared by using (**AccuPower® PreMix Kit**) and this master mix done according to company instructions as following table(3-6):

Table (6): PCR Master Mix Preparation

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

The master mix containing all components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and loading dye). The total volume (20) µL of PCR reaction solution which containing DNA template, two pair of primers and master mix were putting in PCR Eppendorf tube. Then, vortexed in Exispin vortex centrifuge at 3000rpm for 3 minutes, then placed in PCR Thermocycler (MyGene. Bioneer. Korea).

D- PCR Thermocycler Conditions

All PCR reactions were done at same thermocycler conditions by using convential PCR thermocycler system as following table(7):

Table (7): PCR thermocycler conditions by using conventional PCR thermocycler system as following:

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

E- PCR product analysis

The PCR products was analyzed by agarose gel electrophoresis following steps:

1- 1.5% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15

minutes, after that, left to cool 50°C.
2- Then 3µ of ethidium bromide stain were added into agarose gel solution.
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 one well.
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 mA for 1hour.
5- PCR products were visualized by using UV Transilluminator.

-Statistics analysis

Chi –square (x^2) was used for detecting statistical difference of data prevalence of disease and the effect of other factors, the differences were considered statistically significant at $P \leq 0.05$ (Al-Rawi,1989).

Giardia lamblia

Giardiasis according to microscopic examination:

The method was used to detect the *Giardia* spp. trophozoite or cyst, the direct smear by using normal saline (10 %) and lougal iodine. Figure (1), Figure (4-9)

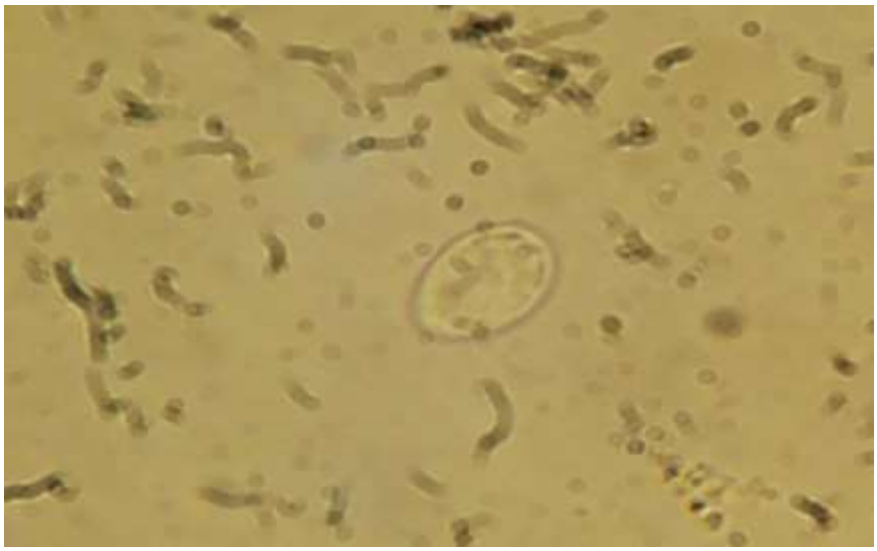


Figure (1) *Giardia lamblia* cyst from camel feces. (magnification x100) by using Floatation method with Zinc Sulfate solution according to the microscopic diagnosis.

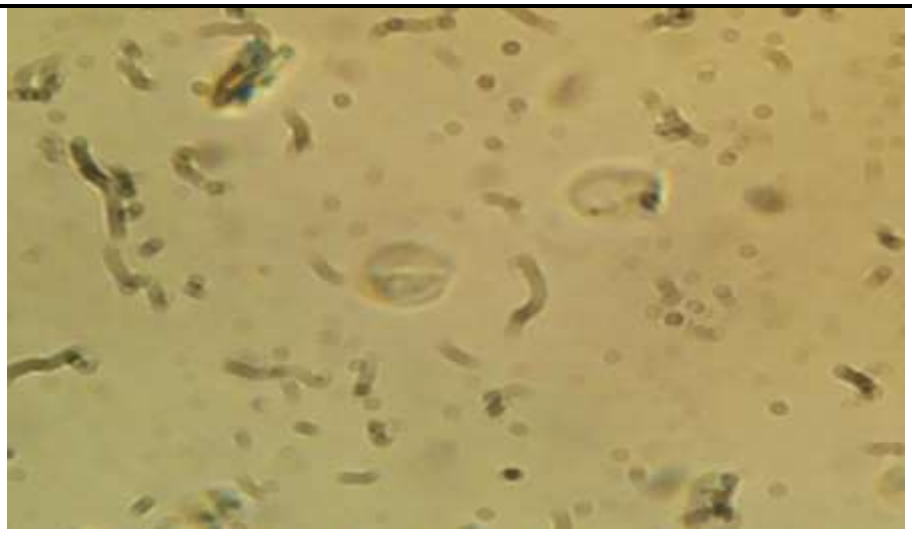


Figure (2) *Giardia lamblia* trophozoite from camel feces show the exon. (magnification x100) by using Floatation method with Zinc Sulfate solution according to the microscopic diagnosis.

-The spread of Giardia infection in the studied areas:

The results of microscopic examination of 100 fecal samples collected from Diwaniyah areas, only 27 (19.3%) are positive, Table (8)

Percentage %	No. of positive samples	No. of fecal samples	Region
19.3% [a]	27	100	Al-Diwaniyah

Similar letters = non-significant differences ($p>0.05$)
Different letters = significant differences ($p<0.05$)
In this study, fecal samples were obtained from camels. Seven months to a year and more in the period from October 2019 - April 2020 in Diwaniyah governorate from various regions and massacres in this governorate, the prevalence rate recorded by microscopic diagnosis according to normal saline solution is 10%, and Iodine in camels (20%)

These results coincide with the finding of Hussain et al (2018) in which the prevalence of giardia infection in camels was reported at 24% according to microscopic examination. It was also agreed with the result of Khashash (2002) in Baghdad, which reported 14.6% of livestock. It corresponds to salami (2005) in Diwaniyah governorate where it recorded 21.41% in cows, Beek et al.(2011) were they showed prevalence (29%) in captive mammals at the zoo of Zagreb, Croatia. Imran *et al.* (2013) in Peshawar, Pakistan were reported 24 positive samples of 150 fecal samples (16%) in ruminants. Chauhan (2004) in Baghdad when he reported 26.4% in cattle too. Khudier (2011) in ThiQar which was observed prevalence 12.9% in cattle. Also Oates *et al.* (2012) observed prevalence rate of *Giardia* infection in domestic and wild animals were 15%.
Because of little studies about this parasite in camels we compared with some ruminants to detect the prevalence of Giardia in animals.

Our results disagreeing with study occurred in Australian calves the prevalence rate were 58% (O'Handly *et al*., 2000). In USA the prevalence in cattle 52% (Trout *et al*., 2005). In Canada other studies reported that the prevalence rate of giardiasis in calves was 70% (Appelbee *et al*., 2003). In Iran the prevalence rate was 37.6% in cattle also (Yousef *et al*., 2011).

These differences between the prevalence of giardiasis in the present study and prevalence of

other studies in the other regions and countries may be related to many factors including environmental changes, number of samples were collected, study season, laboratory methods which used in diagnosis, in addition, the experience of examiner all these factors affect in the final image to infection of *Giardia* (Hamnes *et al*., 2006).

-The prevalence of *Giardia* in microscopic examination according to animal age:

Out of (140) samples positive: 4 (2.9%) less than 1 year and 23(16.4%) more than 1 year in Al-Diwaniyah province. While in Al-Najaf province out of (60) samples positive 2 (22.2%) less than 1 year and 11 (21.5%) more than 1year**.**

Conclusions:

- On the bases of the results, cryptosporidiosis and giardiasis were distributed in camels in Al-Diwaniyah provinces according to Ziehl- Neelsen method and PCR technique.
- Three species recorded in camels *C. parvum*, *C. muris* and *C. andersoni* according to the size of oocysts.
- The first study reported the *C. andersoni* in camels and give the phylogenetic analysis sequences in Iraq (sequences No. KX377971, KX377972, KX377973)
- There are significant difference between age and months of study in camels in *Giardia* infections by microscopic methods.
- Detection of infection and prevalence of *Giardia* by PCR technique.
- Mixed infection was found in different age.
- The phylogenetic study had more accurate in the determination of parasite species.

Recommendations:

- Depending on PCR technique for detection of giardiasis because its faster, sensitive and specific method.
- Identifying the types of Giardia in camels by molecular methods and studying the transmission of isolates to humans.

  beings are crucial to elucidate the potential of zoonotic transmission.
- Other study about parasitic diseases in camels by using phylogenetic analysis to detect the different and similar strain of parasites with neighbored country strain.

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