DETECTION OF *Giardia intestinals* IN HUMAN ,CALVES AND WATER SUPPLIES BY TRADITIONAL AND MOLECULAR METHODS AT BAGHDAD CITY,IRAQ

*** N. S. Alhayali **A.M. Al- Amery * M.H. Hasan**
Assist. Prof. Prof. Assist. Prof.
*** Depart, Microb, Colle, Veter,Med,University of Mosul,Iraq**
**** Depart, Parasit, Colle, Veter, Med, University of Baghdad , Iraq**
Email:nadias.alhayali@gmail.com

ABSTRACT

The current study was conducted during the period from July 2018 to April 2019. One hundred and forty samples from different regions of Baghdad city , Iraq, were collected from 50 calves , 40 children and 50 water supplies for diagnosis of *Giardia intestinalis*. The prevalence of *Giardia intestinalis* in calves, children and water by microscopic examination by using sucrose flotation , 30%, 27.5% and 16% respectively. Twelve positive PCR products were sequenced and deposited in Genebank data base for first time in Iraq, phylogenic analysis demonstrated that 5 sequences belongs to *G.intestinalis* for human(MN244831, MH925331, MN244829, MN244828,MN244830), 3 sequence for calves (MN244827,MH 925332, MN244826), while 4 sequence for water supplies (MH886413, MN244912, MN244910, MN244911).

Key word: *Giardia intestinalis*, calves, children, phylogenetic analysis , gene bank.

التحري عن طفيلي الجيارديا المعوية في الانسان والعجول ومياه الاسالة باستعمال الطرق التقليدية والجزيئية في مدينة بغداد، العراق

***نادية سلطان الحيالي **عامر مرحم العامري *منال حمادي حسن**
استاذ مساعد استاذ استاذ مساعد
***فرع الاحياء المجهرية – كلية الطب البيطري – جامعة الموصل– العراق**
****فرع الطفيليات – كلية الطب البيطري – جامعة بغداد – العراق**

المستخلص

تضمنت هذه الدراسة التي اجريت في الفترة من تموز2018 الى شهر نيسان 2019 جمعت مئة واربعون عينة من مناطق مختلفة من مدينة بغداد تضمنت 50 عينة من العجول، 40 عينة غائط الاطفال ،50 عينة من مياه الاسالة لتشخيص طفيلي *Giardia intestinalis*. في هذه الدراسة تم تسجيل نسب انتشار الطفيلي بواسطة الفحص المجهري بطريقة التطويف السكري ،وكانت النسب 30%، 27.5% و 16% في العجول, الاطفال والماء الجاري على التوالي. تم ارسال اثنتا عشر عزلة موجبة باستخدام الفحص الجزيئي وايداعها في قاعدة بيانات بنك الجينات العالمي، حيث اظهرت نتائج شجرة التطورالوراثي ان خمس عزلات تنتمي الى *G.intestinalis* في الانسان سجلت بالارقام (MN244831, MH925331, MN244829, MN244828,MN244830)، ثلاث عزلات في العجول سجلت بالارقام (MN244827,MH 925332, MN244826) واربع عزلات في المياه الاسالة سجلت بالارقام (MH886413, MN244912, MN244910, MN244911).

كلمات مفتاحية: جيارديا المعوية، عجول، الاطفال، الشجرة الوراثية، بنك الجينات

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INTRODUCTION

Giardia intestinalis is an intestinal protozoan of human and domestic animals causing diarrhea. A lot of outbreaks have been responsible for contamination of raw water by infected cattle especially calves. The main cause of the most outbreaks associated with drinking water, has been identify by deficiencies in water treatment processes (24, 35). The species *G. duodenalis* or *G. intestinalis*, are used in equal frequency in referring to the *Giardia* species, *G. lamblia* is still commonly used to discuss species infecting humans (25). The organism is significant parasite of cattle due its certain pathogenicity (27), its confirmed that the isolates recovered from ruminants are morphologically and antigenically ideal to human isolates (12) and *G. duodenalis* is the only species found in humans and it has a global distribution (34,36). Humans and animals are infected as few as 10 cysts following ingestion of contaminated drinking water, and a symptomatic infection is about 10-100 cysts in human (14). During the 1980s the subject of *Giardia* zoonosis was controversial. *Giardia* isolates depend on morphological properties with six species; such as *G. agilis, G. lamblia G. ardeae, G. muris, G. microti* and *G. psittaci* vary significantly in their biology, host specifity and genetics (17). Recently, the use of molecular tools showed that *G.lamblia* is considered as a specific complex comprising eight major genotypes or assemblages (A – H)(20), from different geographical locations has demonstrated that only *G. duodenalis* genotype E and the zoonotic genotype A and B are associated with cattle infection (16), two of which A and B are found in both animals and humans are associated to have wide host specificity and can be transmitted zoonotically (13), whereas assemblages C, D, E, F and G have strong specificity and strict host ranges (18). Due to need for best understanding of the risk of cattle in the contamination of water sources, decide the prevalence distinguish the isolates of *Giardia* in fecal samples of human and calves and water supplies by using PCR and association with contaminated in the transmission of parasite to human.

MATERIALS AND METHODS

One hundred and forty samples, (40)stool sample from humans,(50) fecal samples from calves and (50) samples water supplies collected from different regions of Baghdad city, Iraq, during from July 2018 to April 2019. All the samples were placed in plastic cup, then transferred directly by icebox to the Department of Parasitology, College of Veterinary Medicine, University of Baghdad. All the samples were stocked at 4Cº and used within 24 h.

Collection of samples

1-Stool samples: forty stool human samples collected from children aged 1-10 years suffered from gastrointestinal discomfort in the hospitals in Baghdad, Iraq

2-Fecal samples of calves : fifty fecal calves samples (1-5 gm) collected directly from the rectum of healthy calves aged under one year.

3- Water samples : fifty water samples (250 ml) collected in a sterile bottle

Microscopic examination

Fecal samples were filtered through a surgical gauze , and floated by sucrose flotation (10). The cysts were collected without adding any preservatives up to 2 weeks at – 20Cº until further use (23).Water sample were brought to the laboratory and left out at room temperature 24 hr. (26) . The positive samples were washed with sterile distilled water without adding any preservative and store at -20 Cº up to 2week .

Genomic DNA extraction

The number of *Giardia* cysts found in this study were 12 samples water = 4, human = 5 and calves = 3, that were positive for *Giardia* on light microscopy. The DNA extraction of *Giardia* was performed from the cysts suspension by using a previously described procedure (26). All the samples were frozen – and thawed six times by using the liquid nitrogen and water bath for 60 seconds in 65Cº. The DNA was isolated from all the suspension by utilizing the kit - G- spin DNA extraction(Intron Biotechnology, cat. No17045, Korea) as manufacturers instructions by using the protocol for the *Giardia*. According to the protocol, as follows:

pipet 200 µl of culture of stool, water placed into a 1.5 ml microcentrifuge tube. Add 20 µl

of proteinase K and 5 µl of RNaseA solution into the sample tube and gently mixed. Then, add 200 µl of buffer BL into the upper sample tube and mixed thoroughly. Incubated the lysate at 56°C for 10 min. For complete lysis, mixed 3 or 4 times during incubation by inverting tube. Centrifuged the 1.5 ml tube briefly to removed drops from the inside of the lid of the tube. Added 200 µl of absolute ethanol into the lysate, and mix thoroughly by inverting 5 - 6 times or by pipetting. After mixing, briefly centrifuged the 1.5 ml tube to eliminate drops from inside of the lid. Carefully apply the mixture resulted to the spin column (in a 2 ml collection tube) without wetting the rim, closed the cap, and centrifuged at 13,000 rpm for 1 min.The DNA in the spin colume was washed by added 700 µl of buffer WA 1 to the spin column, and centrifuged for 1 min at 13,000 rpm. The added of buffer WB 2 (700 µl) was added to the spin column, and centrifuge for 1 min at 13,000 rpm. Discarded the flow-through and place the column into a 2.0 ml collection tube (reuse), Then again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and collection tube altogether. Note: Ensure that 40 (160) ml of absolute ethanol has been added to buffer IWB. The spin column was placed into a new 1.5 ml tube (not supplied), and 30 = 100 µl of the buffer CE directly in to the membrane. Incubated for 1 min at room temperature and then centrifuged for 1 min at 13,000 rpm to elute. The DNA was stored at -20 until use.

Detection of PCR products

Electrophoresis was performed to determine DNA pieces after the processed of extraction or to detected the result of the interaction of PCR during the presence of the standard DNA to detected by visualizing the occurrence of bands in the agarose gel (2%). Prepared of the sgarose gel according to (31,32,33), an Electric current of 7 v\c2 has been exposed for 1-2 h till the tincture has reached to the other side of the gel. The gel has been tested by using a source of the UV with 336 nm after that the gel was staining by putting in pool containing on 30 µl red safe nucleic acid staining solution and then the gel was washing by placing in the distilled water.

Amplification and sequencing

A primary PCR was performed by targeting the *18SrRNA* gene of *Giardia* to confirm the detection of *Giardia* isolated from water, children and calves faeces according PCR (Primer-Blast –NCBI design , Macrogen Korea). A PCR product of 242 bp from target gene was amplified by using forward (5' GCTCTCCCCAAGGACGAAGC- 3') and reverse primer (5'- AGGCGTCGGGCCCTC - 3'). The total volume of the PCR reaction which performed in the present study was 25 µl. The reaction volume containing Taq PCR PreMix (5µl) (Intron/Korea), 1 µl (10 picomols/µl) of each forward and reverse primer, 10µl DNA sample, and 8µl distill water. A total of 35 cycles were carried out, each consisting of initial denaturation 95^{o}C for 5 min, while denaturation -2 95^{o}C for 45 s , annealing 62^{o}C for 45 s , and extension-1 72^{o}C for 45 s , finally extension-2 72^{o}C for 10 min . The DNA ladder (KK6302) (Kapa/ USA) was used to detect the band size of target DNA.

RERSULTS AND DISCUSSION

The result of this study declared that the *Giardia intestinalis* cysts were detected microscopically only the 34 samples from the 140 samples collected from calves, human, and water (Figure 1). The high percentage of infection had found in calves 30% (15/50), while it found in the children and water were 27.5% (11/40) and 16% (8/50), respectively (Table1).

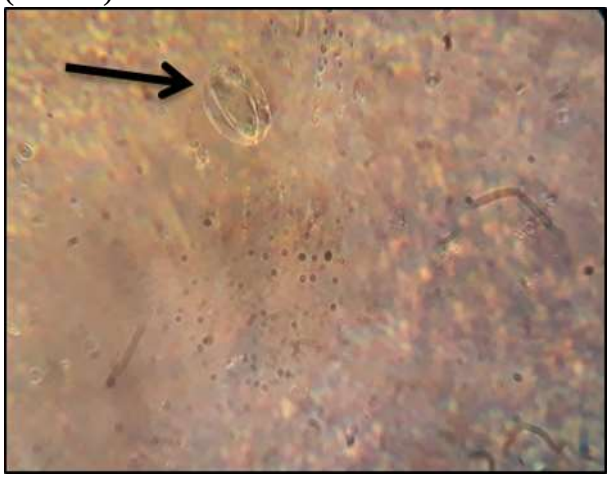

Figure 1. *Giardia spp*. cysts from calf fecal sample by flotation method((100X).

Table 1 . Detection of cyst of *Giardia intestinalis* by microscopic examination .

Samples	No. of samples examination	SampleS positive No. (%)
Calves	50	15 (30%)
Children	40	11 (27.5%)
Water supplies	50	8 (16%)
Total	140	34(24.28%)

In this study, the prevalence rate of *G. intestinalis* in calves was 30.0%. Other studies showed with lower rate of infection compared with the present results like (28,5), which were 5.45% and 10.27% respectively. Our results was similar to the result of (3) which is recorded the percentage of infection 30.6% in cow in Basrah province, while our studies recorded a highest rate of infection in cattle like (4), which recorded 70% in cattle in Al-Qdisiya governorate in Iraq. In the studies done in the neighboring countries like Iran and Turkey,9.34% of apparently healthy calves in Iran (23)and 14.7% in Turkey (19) .This is what confirmed by(37), that risk factor of infection reduce with the age of the animal and diverse by the season of sampling. The differences between the prevalence rates of infection with *G. intestinalis* in present study and prevalence rate of other studies in other regional countries, may be due to several factors including, number of samples collected, environmental contaminated study season, laboratory methods which were used in diagnosis and age of hosts examined(4). The prevalence rate of *Giardia intestinalis* in children was 27.5% in Baghdad city, this result is similar to the different studies in Iraq(7,1, 6).The results are also in coincidence with studies in other parts of the world (15,22). The high infection rate properly due to protein –energy malnutrition, vitamin A deficiency, anemia, mineral deficiency (particularly iron and zinc),poor cognitive and educational level. In addition , there are several socioeconomic factors that have been identified as important risk factors correlating with *Giardia* infection, such as illiteracy, absence of adequate sanitation and water treatment systems, indigence and poor hygienic(9) *Giardia* cysts have been separated from water supplies in several parts of the world (38,11), therefore, this study focused on the running water because it is a major source of infection for human and animals. This confirms (30) that 90% of announce outbreaks of these pathogenic protozoans occur through water while 10% are regarding to food. The results as showed the presence of *Giardia* cysts in samples of water supplies at percentage 16%. This finding is coincide with the result of Al saqur *et al* (8) concerning presence of *Giardia* cysts in tap water at percentage 3.47%. Among the different causes which may show the high incidence among consumers of water, the cysts of *G. intestinalis* relatively resistant to disinfectant chlorine (21) Most of studies showed the *Giardia* sp. can infect human and animals by swallowing contaminated water from parks, lakes, streams, rivers, ponds during the swimming and playing in these places (2) this confirmed by other results in current studies that the running water is probably the main source of infection with cysts of *Giardia Intestinalis* for human and animals. The researcher (26) added that the high infection rate in water and deficit of water sort should be resolved by water organization responders, it is strongly recommended to use home filtration systems for consumptions of safe water. According to the PCR technique, all the *Giardia intestinalis* in this study were positive and the molecular weight of the *Giardia intestinalis* serotypes was 242 bp (Figure 2). In addition, the result of the microscopic examination was concorded with the result of the PCR result.

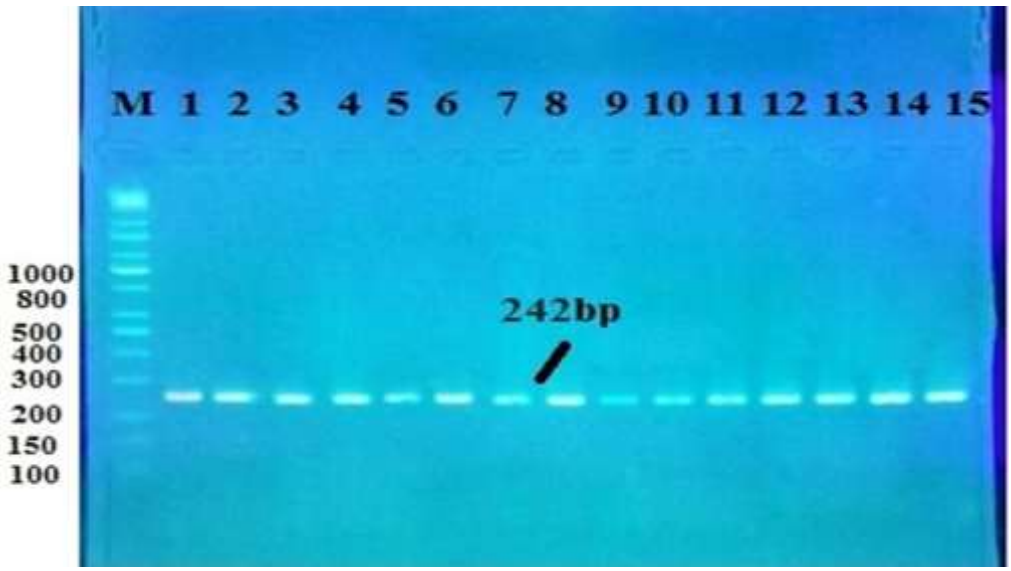

Figure 2. Agarose gel electrophoresis (2%) display the typical amplicon of DNA product of *Giardias* serotypes. The amplification of DNA appears as a ladder-like pattern. Lane 1 is the positive control, Lanes 2–6 represent positive for *G. intestinalis* of Homo sapiens, Lanes 7-10 represent positive for product for *G. intestinalis* of water, and Lanes 11-13 represent for *G. intestinalis* of calves. Lane M are DNA Marker 100 bp ladder (Kapa /USA).

It was registered globally in the website NCBI in the following numbers.ID for *G.intestenalis* children(MN244831,MH925331, MN244829, MN244828,MN244830).IDFor *G. intestinalis* for calves (MN244827, MH925332, MN244826). ID for *G. intestinalis* for water supplies(MH886413,MN244912, MN244910, MN244911). Based on the phylogenetic tree, there is a relationship between the *Giardia intestinalis* had found in the present study. On one hand, the percent of a relationship between *Giardia intestinalis* isolated from children (MN244831.1) with *Giardia intestinalis* isolate from water supplies (MN244910.1, MN244911.1) was 99%. On another hand, the percent of a relationship between *Giardia intestinalis* isolated from children (MH925331.1) with *Giardia intestinalis* isolate from calves (MN244827.1) was 97%(figure 3) .

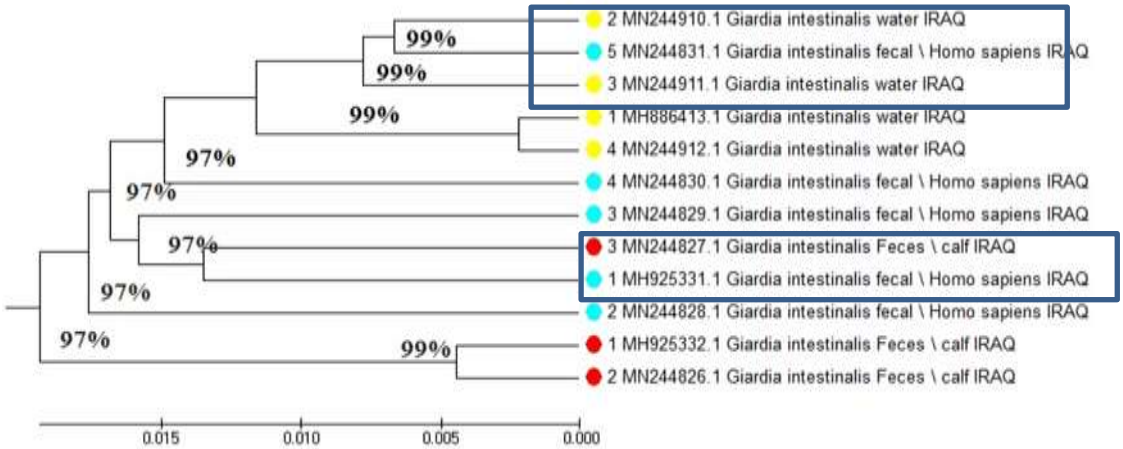

Figure 3. Phylogenetic tree. The evolutionary distances analyses were conducted in Mega 6 and NCBI. Red spot: *Giardia.intestinalis* strain isolated from calf, Green spot: *Giardia. intestinalis* strain isolated from children, yellow spots: *Giardia. intestinalis* strain isolate from water supplies

In addition, *Giardia intestinalis* isolated from Turkey (KX384157.1) and Australia (AF113899.1) revealed a relationship with the *Giardia intestinalis* isolated from the children (MN244829.1) (97%). While, there is no relationship between the *G. intestinals* isolated in this study with *G. intestinals* isolated from Uganda, Brasil, China , Egypt, and Saudi Arabia(figure4).

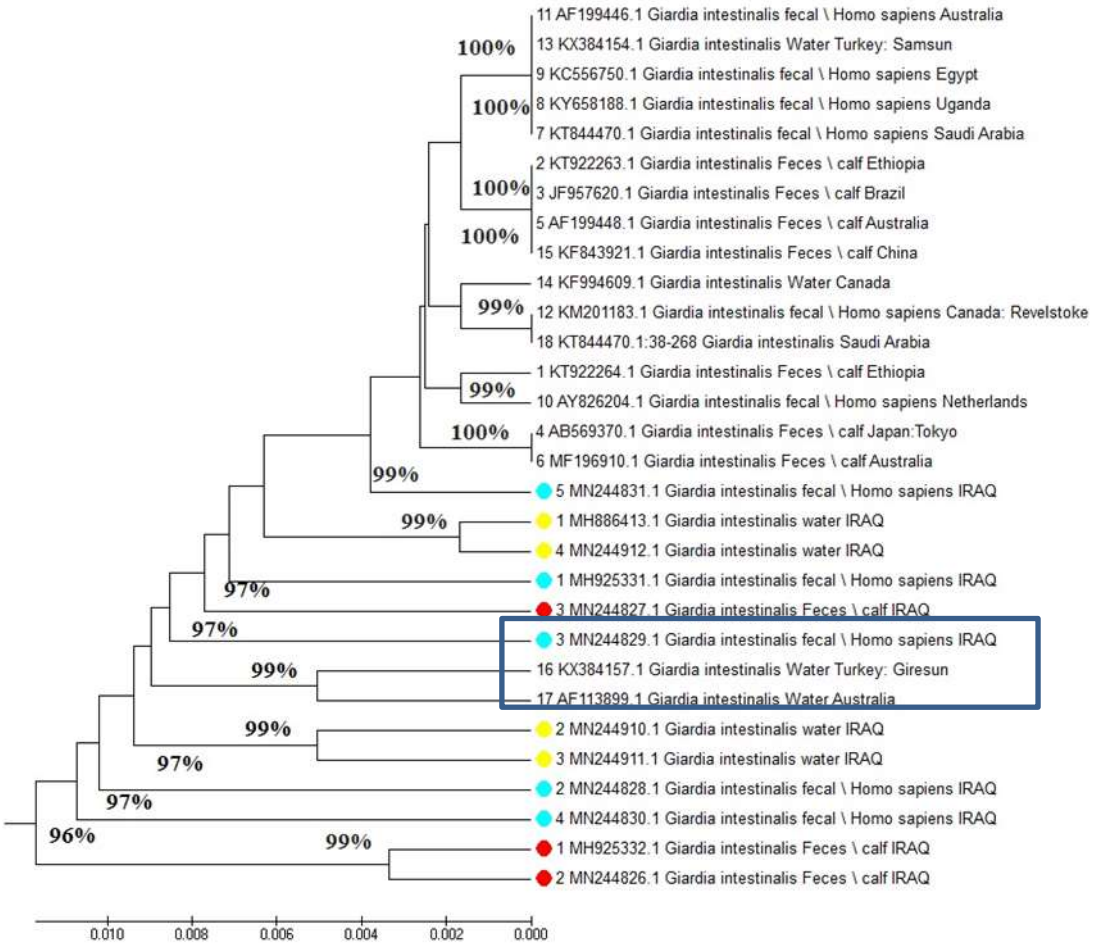

Figure 4. the phylogenetic tree using neighbor joining method. The evolutionary distances analyses were conducted in Mega 6 and NCBI. Red spot: *G.intestinalis* strain isolate from calf, Green spot: *G. intestinalis* strain isolated from children , yellow spots: *G. intestinalis* strain isolated from water supplies

In this study, simple PCR assays with primer specific *18 S r RNA* have been applied for the detection of *Giardia* isolates in human, calves faecal and water specimen. It showed that molecular weight of *Giardia intestinalis* isolated from the human, calve and water was 242 bp. According to the phylogenetic tree (between the Iraqi genotype), the *Giardia intestinalis* isolated from the human , animals and water showed the relationship between them. The *Giardia* which was isolated from 5MN244831 in human matches with *Giardia intestinalis* isolated from water Accession No : MN244910, MN244911 respectively with an identical percentage 99%. In addition the *Giardia* percentage of relationship of *Giardia* isolated from faeces of calves MN244827 with *Giardia intestinalis* in the stool of human sample MH925331was 97%. Cattle particularly calves are an significant source of infection for human and part an important role in environmental contamination by excreting large quantities of cysts into the environment and contaminate water sources used for animals and human consumption. Since conventional methods of water treatment reduce these parasites, it is better to understand the significance of cattle in the contamination of water source on farms (29), this confirmed by (27)their results that calves shed *Giardia* cysts potentially infectious to humans. Researchers The phylogenetic tree depending on the neighboring country from Turkey shows matching percentage of 99% of *Giardia intestinalis*.

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