Occurrence of Cystoisospora Species, Based on rRNA ITS1 Locus, in Cats in Babylon City of Iraq

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Abstract

This study was conducted to uncover the molecular prevalence of Cystoisospora species based on the rRNA ITS1 locus, in cats in Babylon city, Iraq for a period of 6 months from the beginning of November 2021 to the end of April 2022. A total of 100 cat's faecal samples were examined by Polymerase Chain Reaction (PCR). Our data disclosed that 22 out of 100 cats (22%) were shown to be infected with Cystoisospora felis. The parasite burden in males was 26% (13/50) and it was higher than females 18% (9/50). Kittens under 6 months of age had the highest infection rate 34.78% compare to other age groups. Additionally, the months had also a comparable effect on the infection rate with no significant difference, where the highest infection rate was 34.78% and recorded in November and the lowest was 6.66% and recorded in March. Database search showed sequence homology with the Australian C. felis, Chinese C. felis and the Australian Cystoisospora spp. isolates. This study exhibited that the C. felis is the most common species in cats in Iraq in comparison to C. rivolta.

Keywords: *Cystoisosporiasis, parasite, ITS1 locus C. felis, C. rivolta, PCR.*

Introduction

Cystoisosporiasis, previously known as isosporiasis, is an intestinal disease caused by a protozoan parasite called Cystoisospora (former name Isospora). It is worldwide distributed, especially in tropical and subtropical areas infecting both man and some animals (12). Cats are one of the main definitive hosts of this parasite and get infected with Cystoisospora felis and Cystoisospora rivolta through the ingestion of sporulated oocysts with food or water or tissue cysts from infected paratenic hosts. Some studies refereed to ability of infection through the ingestion of paratenic hosts that have eaten sporulated oocysts such as flies, dung beetlesor sometimes cock-roaches (13). As in other parasites such as Toxoplasma, the clinical disease associated with Cystoisospora infections occurs most commonly in young, debilitated and immunosuppressed cats (13, 18, 22). Cats with or without clinical signs of disease can pass oocysts, so the finding of oocysts does not prove a disease association (26). Cystoisospora felis is commonly believed to induce diarrhea in kittens but few experimental data are available from young adult cats. Phylogenetic analysis of the 18S rRNA has grouped Cystoisospora together with Toxoplasma, Neospora and Sarcocystis than Eimeria. This suggests that Cystoisospora belongs to the family Sarcocystidae instead of Eimeriida (17, 28, 30). Various epidemiological studies have investigated the

occurrence of Cystoisospora in cats across the world. As reported in many studies the parasite infected 16.4% of cats in Greece (35), 7% in India (11), 43% in Kenya(29), 14% in Canada (37) and 5.1% in Mexico(25).Based on the parasite species, the differentiation between C. felis andC. rivoltacan be easily done through the morphology of the oocyst and it has been reported by many researchers among different countries; China (39), Egypt (1) and Iran (23).Furthermore, in Iraq, several studies have been conducted described infection with Cystoisospora in cats (4, 7, 16).

In the vast majority of cities inIraq, cats usually move freely in neighborhoods and inside homes, where they prey mice and rodents. Since cats are more prevalent in urban residential areas and have easy access to the environment, they may contaminate soil, food, and water, which could lead to more parasite infections. As most of studies have emphasized on zoonotic parasites of cats, other parasites have received little attention in Iraq, the current study was designed to predict the prevalence of Cystoisospora in cats in Babylon, 120 Km Ssouth of Iraq.

Materials and Methods

Faecal samples collection and processing

The study was conducted on cats brought to the veterinary clinics in Babylon province from November 2021 to April 2022. A total of 100 fecal samples were randomly collected from cats, taking into consideration the age, sex and the month of collection. A minimum of 5 g of feces was directly collected and immediately placed into a plastic container, and stored at 4°C. Some animals showed symptoms of diarrheaoea at the time of feces collectionn. Collected samples were examined microscopically by direct smear, and incubated with 2.5% of potassium dichromate at room temperature for sporulation and identification (3, 5). Finally, the identified oocysts were purified from samples using NaCl flotation method (8, 14, 20) and kept in 4°C for the next experiment.

rRNA ITS1 locus amplification and sequencing

DNA extraction was performed using AccuPower PCR Premix Kit (Bioneer, South Korea) and according to the manufacturer's instructions. DNA was extracted directly from oocysts obtained by flotation method (NaCl method). Amplification was done for molecular identification of Cystoisospora using primers mentioned by Samarasinghe et al. (31). However, because of non specific bands that have been detected by these primers, additional PCR analysis has been conducted using different set of primers. Briefly, amplification of 225 bp of rRNA ITS1 locus of Cystoisospora (MK430064.1) was carried using a primer set of ITS1-fb3’ CTA CTG AAT CCC ATA ATC AGG AC’5 and ITS1-rp 3’CCA AAA TCT CAA GGA GAT AGG AG’5 as previously described by Scorza et al. (33). PCR was carried out using Thermal Cycler Veriti (Thermo Fisher Scientific, USA) and the following parameters: Initial denaturation step of 95◦C for 5 min, 30 cycles of denaturation at 95◦C for 45s, annealing at 54◦C for 45 s and extension at 72□C for 1 min, and a final extension of 72□C for 7 min. All the PCR products were analysed by electrophoresis on agarose gel, stained with RedSafe (Intron, Korea), and visualized on UV transilluminator. After that, amplicons were sent for sequencing and similarity search was performed by the searching sequences in the National Centre for Biotechnology information (NCBI). Moreover, multiple sequence alignment of the partial ITS1 locus sequence was created and sequences were

aligned by MSCLE tool on SnapGen software (Version 5.0.5. Iowa University, USA). Phylogenetic tree based on Neighbour-joining analyses with 500 bootstrap replicates were performed using the MEGA X software.

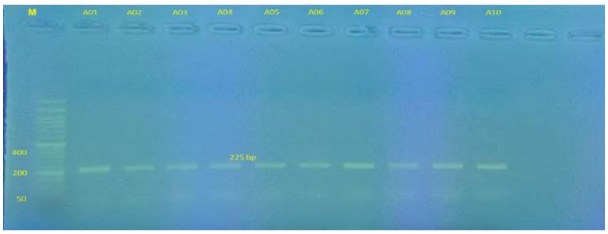
Statistical analysis

SAS program was used for sstatistical analysis to show the significant comparisons between percentages (33).

Results and Discussion

To date, Cystoisospora spp. species have usually been recognized depending on the oocyst morphology, however, DNA confirmation of the species have been rarely used. Therefore, the GenBank has insufficient sequencing data for Cystoisospora spp. for the 18S rRNA and rRNA ITS1 loci. In the present study, we amplified DNA of Cystoisospora from oocysts in the feces of cats using rRNA ITS1 locus. The partial sequence of the rRNA ITS1 locus was amplified directly from the genomic DNA of Cystoisospora parasite. Gel electrophoresis analysis showd the band of the amplified gen with the predicted size of 200 bp (225 bp) (Figure 1). Although, oocysts were detected microscopically by direct smear (data were not shown), no bands were obtained using Cystoisospora specific primers. The reason is still unknown, it might be the few numbers of oocysts used for DNA extraction (10-100 oocysts),while Oliveira et al. (31) have used 3-7 x 107 oocysts. However, several studies obtained DNA form single oocyst (26,27). Consequently, two other set of primer designed by Scorza et al. (34) who successfully amplified 225 bp of rRNA ITS1 locus of Cystoisospora from 10-100 oocysts.

Figure. 1. Gel electrophoresis of the partial region of ITS1 rRNA locus of C. feliswith an expected band of 225 bp (Lanes A01-A10).M: DNA ladder (Safe-Green 100bp Opti-DNA Marker).The gel was 1.5% and the DNA dye is RedSafe V: 90, Time: 45 min



Our data revealed that the overall prevalence of Cystoisospora in cat's faeces was 22%. Of the 100 fecal samples included in the study, 26% (13/50) males and 18% (9/50) females were shown to be infected, with no significant difference($P>0.05$) (Table 1).This percentage is close to several studies around the world. In a study in Greece, 189 out of 1150 cats were found infected with this parasite with an overall infection rate of 16.4% (35). Likewise, in Canada, a group of researchers were sought to study the faecal parasites in dogs and cats and the prevalence of Cystoisospora was 14.7% (37). However, in several Iraqi studies, the prevalence was 20.78% in the North of the country (16), though, 6.92% of cats were found infected in the South (4) and 6.6% in the middle, in Baghdad city (7). Surprisingly, the prevalence was as high as 80% in India (11) and as low as 1% in Turkey (21) and this variation may be attributed to the difference in the people culture and governmental applied hygienic measures in these countries. Usually developed countries show low infection rate compare to developing and poor countries due to lack of hygiene and sanitation standards, and socioeconomic conditions. Limited studies were carried out on cat's Cystoisospora in European countries, where the infection rate in

Austria, France, Hungary, Italy, Romania and Spain was 4-8% only (10, 15). Cystoisosporais more frequent in young ages than in adults, where kittens show more susceptibility and excrete oocysts as early as week three of age (9). We noticed that the higher infection rate was at the age group of ≤ 6 months and the lowest was at the age group of 6-12 months (Table 2). Young kittens at the age group ≤ 6 months exhibited the highest infection rate at 34.78%, while the lowest was 10% at the age group of 6-12 months. Most of the studies worldwide indicated that the age is an important risk factor and influence the burden of Cystoisospora and the age group of ≤12 months are the most vulnerable. The prevalence in the European union ranged from 38% at the age group of ≤ 6 months (10) and 64% at the age group 6-12 months (15). Similar findings were reported from Egypt, namely 32% of cats ≤ 7 months were found infected with one of Cystoisospora species (1). However, the infection rates are, as low as 13% and 14% in Iraq (16), and still affecting the age group ≤ 6 months.The prevalence of Cystoisospora parasite varies conferring with the season and the geographical area. Where the shirt-sleeve temperature (23-30□C) and moderate humidity are the optimal conditions for the parasite development (12). In this study, the burden of the parasites was at the peak 34.78% (8/23) in November and the bottom 6.66% (1/15) in March, with no significance difference (P>0.05) (Table 3). Our data are close to Tzannes et al. (35) and Al-Taie and Al-Muhsin (7) where recorded the highest infection rate in October-December. However, some risk factors such as sex and season are no longer included in the most recent studies except in some studies from the third-world countries such as Egypt, Iraq and Iran.

Table 1. The infection rate of Cystoisospora in accordance with sex

Sex	No. of Samples	Positives	Percentage %
Males	50	13	26
Females	50	9	18
Total	100	22	22

Table 2. The infection rate of Cystoisospora in accordance with the age

Age	No. of samples	Positives	Percentage %
≤ 6 M	23	8	34.78
6-12 M	50	5	10
≥ 1 Y	27	9	33.33

*P-VALUE 0.015 *CHI-SQUARE 8.407

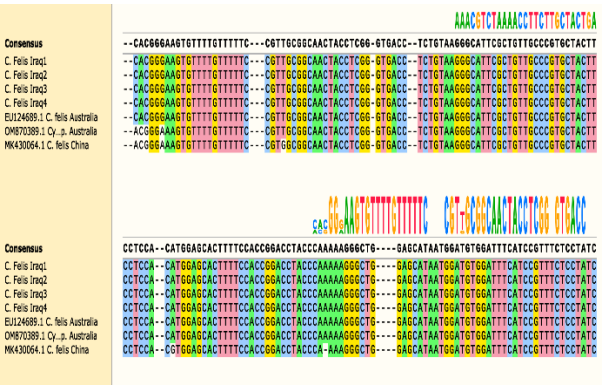
Table 3. The infection rate of Cystoisospora in accordance with the months of the study

Month	No. of Samples	Positives	Percentage %
November	23	8	34.78
December	17	4	23.52
January	20	2	10
February	7	2	28,57
March	15	1	6.66
April	18	5	27.77
Total	100	22	22

Cystoisospora species can be easily differentiated based on morphology, where C. felis oocyst is large in size measures 42-43 x 31-33 µm and C. rivolta is medium size, 25.4 x 23.4 µm in diameter (13). In our study, microscopical analysis between C. felis and C. rivolta and the infection rate with the former was revealed higher than recognised only in 4 samples out of 120 (data not shown). Therefore, these four C. rivolta samples might not have been selected in the random 100 PCR samples out of 120 total samples. Database search sequence alignment (Figure 2) and phylogenetic tree (Figure 3) revealed that our

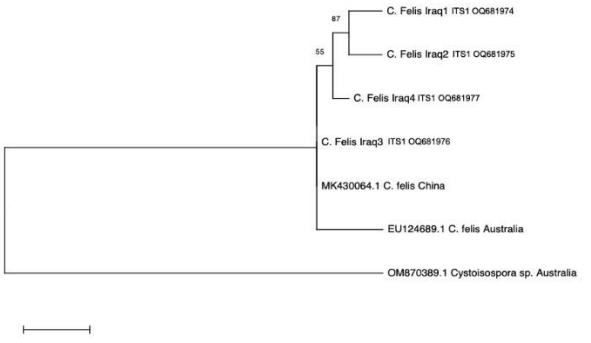
isolates are highly similar with the Australian C. felis isolate (EU124689.1),Chinese isolates were submitted to the GenBank and were given following accession numbers; C.felis_Iraq1_ITS1 (OQ681974), C.felis_Iraq2_ITS1 (OQ681975), C.felis_Iraq3_ITS1 (OQ681976), C.felis_Iraq4_ITS1 (OQ681977).

Figure 2. Multiple sequence alignment of C. felis Iraqi isolates, aligned against the sequence of C. felis from Australia and China obtained from the GenBank. Each colour represents type of nucleotide, changes in the nucleotides are marked as uncoloured, dashed (-) were created during alignment process. Sequence created by SnapGen



Report from around the world revealed that C. felis is more dominant than C. rivolta such as in China (39), Egypt (1) and Iran (23) and other European countries (10). However, only one study in Portugal by (37) reported high infection rate with C. rivolta. In brief, although, cats in Iraq are infected with different gastrointestinal parasites, the infection with Cystoisospora represents is the most common comparing to other parasites. Our findings indicated that Iraq recorded the lowest infection rate among other countries, which might indicate the owners are well-educated and has high level of awareness about the risk of cat's parasites and cats are highly likely to receive veterinary care compare to other animals.

Figure 3. Phylogenetic relationships between Cystoisospora felis Iraqi isolates and C. felis from Australia and China, inferred by neighbour-joining test of rRNA ITS1 locus. Bootstrap values of 500 isolates for neighbour-joining analysis are disclosed



Reference

1. Abbas, I., M. A. l. Araby, B. Elmishmishy, E. Sayed and E.Alfy.2022. Gastrointestinal parasites of cats in Egypt: high prevalence high zoonotic risk. BMC. Vet. Res.18:420.

2. Al-alousi, T. I., and O. I. Mahmood.2012. Detection of Cryptosporidium oocysts in calves and children in Mosul, Iraq. The Iraqi Journal of Veterinary Medicine, 36(0E): 280–285.

3. Al-Amery, A. R. and A. M. A. Al-Amery .2020. Molecular diagnosisof Cryptosporidium spp in waterbuffaloes in Babylon province, Iraq. Iraqi J. Agric. Sci., 53(1):147–56.

4- Al-Aredhi, H. S. 2015. Prevalence of gastrointestinal parasites in domestic cats (Felis catus) in Al-Diwaniya province/Iraq. Int.J.Curr.Microbiol.App.Sci.4. (5):166-171.

5- Alhayali, N. S., A. M. Al- Amery. 2019. Detection of Giardia intestinalis in human, calves and water supplies by traditional and molecular methods in Baghdad city. Iraq. Iraqi J. Agric. Sci. 51(5):1428-1435

6- Al-Jassim, K. B.N., Y. S Mahmmod, Z. M. Salem, and A.Al-Jubury. 2017.Epidemiologicalinvestigation of gastrointestinal parasites in dog populations in Basra province, Southern Iraq. J. Parasit. Dis., 41(4):1006–13.

7- Al-Taie, D. A.and F. A. Al-Muhsin. 2022. A comparative study of some intestinal parasites in fecal samples of domestic and stray cats in Baghdad, Iraqi. Comparative Parasitology. 89(1): 31-35.

8- Al-Zubaidi, M. T. S., L. I. Kadhim and Z. I. Ibrahim. 2018. Incidence and experimental infection of Cryptosporidium in baileyi chickens. Iraqi J. Agric. Sci.49(2):269–278.

9- Barutzki, D. 2013. Endoparasites in young dogs and cats up to one year of age. parasitol.Res.,112:S119–S131

10- Beugnet, F., P. Bourdeau, K.Chalvet-monfray, V. Cozma, R.Farkasand J.Guillot. 2014. Parasites of domestic owned cats in Europe: co-infestations and risk factors.Parasites and Vectors, 7:291.

11- Borkataki, S., R.Katoch, P. Goswami, R. Godara, J.K. Khajuria andA. Yadav. 2013. Prevalence of parasitic infections of stray cats in Jammu, India. Sokoto Journal of Veterinary Sciences. 11(1): 1-6.

12- Dubey, J. P. 2018. A review of Cystoisospora felis and C. rivolta-induced coccidiosis in cats. Vet. Parasitol. S0304-4017(18)30337-6.

13- Dubey J. P. 2020. Coccidiosis in livestock, poultry, companion animals, and humans. CRC PressTaylor and Francis Group, NY, USA

14- Faraj, A. A.2019.Traditionalandmolecularstudyof Cryptosporidium in domestic dogs in Baghdad city, Iraq. Iraqi J. Agric. Sci. 50(4):1094–1099.

15- Giannelli, A., A. Capelli, A. Joachim, B. Hinney, B. Losson, Z. Kirkova, M. René-Martellet, E. Papadopoulos, R. Farkas, E. Napoli, E. Brianti, C. Tamponi, A. Varcasia, A. M. Alho, L. M. de Carvalho, L. Cardoso, C. Maia, C. Mircean, A. D. Mihalca, G. Miró, M. Schnyder, C. Cantacessi, Colella, M. A. Cavalera, M. S. Latrofa, G. Annoscia, M. Knaus, L. Halos, F. Beugnet, andD.Otranto.2017. Lungworms and gastrointestinal parasites of domestic cats: a European perspective.Int.J. Parasitol.47(9):517–28.

16- Hassan, H. F., and A. K. R. A. barzinji.2018.epidemiologicalsurvey on stray dogs and cats'gastrointestinal parasites in Kirkuk province, Iraq. Kirkuk University Journal, 13(1): 228-238.

17- He, P., J. Li, P. Gong, J. Huang and X. Zhang. 2012. Veterinary Parasitology Cystoisosporaspp. from dogs in China and phylogenetic analysis of its 18S and ITS1 gene. Vet. Parasitol.190(1–2):254–258.

18- Jalil, D. R and J. A. Mohammed.2014. Sero-pathological Diagnosis of Toxoplasma gondii in Stray Cats in Baghdad Province. The Iraqi Journal of Veterinary Medicine. 38(1): 92–98.

19- Kallo, O. J. 2002. Detection of Toxoplasmosis in cats and sheep. The Iraqi Journal of Veterinary Medicine. 26(1): 159–164.

20- Kareem, S. M., and Kawan, M. H. (2020). Traditional Diagnosis of Eimeria spp. in Fallow Deer at Middle Parts of Iraq. The

Iraqi Journal of Veterinary Medicine, 44((E0), 94–99. https://doi.org/10.30539/ijvm.v44i(E0).1028

21- Karakavuk, M., N. Selİm, B. Yeşİlşİraz, G. E.Atliand Ö. N. Alan. 2021. Prevalence of gastrointestinal parasites in stray cats of Izmir Turkey. Animal Health Prod and Hyg.10 (1): 6 - 11.

22- Kh. Rifaat, L., and Z. Jawdat, S. 2021.Prevalence of Toxoplasmosisamong sheep and goat in Baghdad area. The Iraqi Journal of Veterinary Medicine, 22(1),43–49.

23- Khademvatan,S.,R. Abdizadeh,F. Rahim and M. Hashemitabar.2014. Stray cats' gastrointestinal parasites and its association with Public Health in Ahvaz City, South Western of Iran. Microbiol. 7(8):e11079

24- Legua, P. and C. Seas. 2013. Cystoisospora and Cyclospora. current opinion in infectious diseases. 26(5): 479-483.

25- Libertad, T., I. Cossío, A. Danae, M. Luna, M. R. Mejia and C. R. Núñez. 2021. Risk factors associated with cat parasites in a feline medical center. Journal of Feline Medicine and Surgery Open Reports. 1- 9.

26- Lindsay, D. S., A. E. Houk, S. M.Mitchell and J. P. Dubey. 2014. Developmental biology of Cystoisospora(Apicomplexa:Sarcocystidae) Monozoic Tissue Cysts. J.Parasitol. 100(4):392–398.

27- Matsubayashi,M., H.Takayama, M. Kusumoto, M. Murata, Y. Uchiyama and M. Kaji. 2016. First report of molecular identification of Cystoisosporasuis in piglets with lethal diarrhea in Japan. Acta Parasitologica. 61(2):406–411.

28- Matsubayashi, M., R. A. Carreno, H. Tani, R. Yoshiuchi, T. Kanai andI.Kimata. 2011. Phylogenetic identification of Cystoisospora spp. from dogs, cats, and raccoon dogs in Japan. Vet. Parasitol.176(2–3):270–274.

29- Njuguna ,A. N., J. M. Kagira, S. M. Karanja, M. Ngotho, L.Mutharia and N. W. Maina. 2017. Prevalence of Toxoplasma gondii and other gastrointestinal parasites in domestic cats from households in Thika Region, Kenya. BioMed. Research International, 2017(76158100):1-6

30- Ogedengbe M. E., J. D. Ogedengbe, J. C. Whale, K. Elliot, M. A. Juárez-estrada and J. R. Barta.2021.Molecular phylogenetic analyses of tissue coccidian (Sarcocystidae; apicomplexa) based on nuclear 18s RDNA and mitochondrial COI sequences con fi rms the paraphyly of the genus Hammondia. Parasitol. Open. 2(e2):1–16.

31- Oliveira, U. C., J. S. Fraga, D. Licois, M. Pakandl and A. Gruber. 2011. Veterinary Parasitology development of molecular assays for the identification of the 11 Eimeria species of the domestic rabbi(Oryctolagus cuniculus).Vet. Parasitol.176 (2–3):275–80.

32- Samarasinghe, B., J. Johnson and U.Ryan. 2008. Phylogenetic analysis of Cystoisospora species at the rRNA ITS1 locus and development of a PCR-RFLP assay. Experimental Parasitology. 118:592–595.

33- Al-Gharban, H.A.A.J. (2017). Seroepidemiological detection and culture utilization for diagnosis of carrier horses and donkeys with strangles. Journal of Education College Wasit University, 1(28), 649-660.

34- Scorza, A. V., P. Tyrrell, S.Wennogle, R. Chandrashekar and M. R. Lappin. 2021. Experimental infection of cats with Cystoisospora felis. J. Vet. Intern Med.35:269–272.

35- Symeonidou, I., A. I. Gelasakis,k.Arsenopoulos,A. Angelou,F.Beugnet and E. Papadopou-los .2018.Feline gastrointestinalparasitism in Greece:emergentzoonotic species and associatedrisk factors. Parasite and vector11(277):1–13.

36- Tzannes, S., D. J. Batchelor, P. A. Graham, G. L. Pinchbeck, W.Wastling and A. j. German. 2007. Prevalence of Cryptosporidium, Giardia and Isospora species infections in pet cats with clinical signs of gastrointestinal disease. journal of feline medicine and Surgery.10,1e8.

37- Villeneuve, A. et al. (2015) 'Parasite prevalence in fecal samples from shelter dogs and cats across the Canadian provinces. Parasites and Vectors,(8):281-290

38- Waap ,H. , J. Gomes and T. Nunes.2013. Parasite communities in stray cat populations from Lisbon, Portugal. Journal of Helminthology. 88: 389–395.

39- Yang, Y. and H.Liang. 2015.Prevalence and Risk Factors of intestinal parasites in cats from China.BioMed.Research International. (967238):1-5.