MICROSCOPIC AND MOLECULAR DIAGNOSIS OF *Ascaridia* spp. IN DOMESTIC PIGEONS(*Columba livia domestica*) IN BAGHDAD CITY,IRAQ

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ABSTRACT

Ascaridiosis is a very important parasitic disease of birds, it is caused by *Ascaridia*. This study was conducted to identify the *Ascaridia* species by microscopic and molecular assay in Baghdad city. One hundred and sixty fecal samples were collected from domestic pigeons during the period from 1/1/ 2019 to 31/3/ 2019. Results showed that the rate of infection for *Ascaridia* spp. 15.62% by microscopic examination. Significant difference was observed in infection rates between males and females pigeons. Fifty samples randomly selected and subjected to molecular diagnosis of *Ascaridia* spp.. Molecular examination results, the total infection rate showed 16%(8/50). The eight positive PCR products were sequenced and deposited in Gene bank data base, phylogenic analysis demonstrated that 4 sequences belongs to *Ascaridia galli* (MK918635.1, MK918636.1, MK918847.1, MK919081.1), while 2 (MK919199.1, MK919200.1) belong to *Ascaridia nymphii* and 2 (MK919207.1, MK919264.1) belong to *Ascaridia numidae*. It is the first study in Iraq to diagnosis of *Ascaridia nymphii* and *Ascaridia numidae* in domesticed pigeons by using conventional PCR.

Key word: molecular techniques, phylogenic analysis, ascaridiosis, pigeons.

مجلة العلوم الزراعية العراقية -2020 :51 (4):1220-1225 فرج والعامري

التشخيص المجهري والجزيئي لطفيلي *Ascaridia spp* في الحمام الداجن (*Columba livia domestica*) في مدينة بغداد، العراق

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مرض الاسكارس من الامراض الطفيلية المهمة في الطيور، يتسبب عن الاصابة بطفيلي *Ascaridia* . اجريت هذة الدراسة لتحديد انواع الطفيلي ال *Ascaridia* بواسطة الفحص المجهري والجزيئي في مدينة بغداد. جمعت 160 عينة براز من الحمام الداجن خلال المدة من 2019/1/1 ولغاية 2019/3/31 .اظهرت النتائج نسبة الاصابة بطفيلي .*Ascaridia* spp 15.62% باستخدام الفحص المجهري .ولوحظ فرق معنوي في نسبة الاصابة بين ذكور واناث الحمام . انتخب 50 عينة براز عشوائيا لاجراء الفحص الجزيئي لتشخيص انواع طفيلي ال *Ascaridia* . أظهرت نتائج الفحص بتقنية تفاعل سلسلة البلمرة نسبة اصابة الكلية بلغت 16%(50/8). ارسل ثمان عزلات موجبة باستخدام الفحص الجزيئي ولايداعها في قاعدة بيانات بنك الجينات العالمي، حيث اظهرت نتائج شجرة التطور الوراثي أن 4 عزلات تنتمي *إل Ascaridia galli* سجلت بالارقام (MK918635.1 , MK918636.1, MK918847.1, MK919081.1) و2 عزلات تنتمي إلى *Ascaridia nymphii* سجلت بالارقام (MK919199.1, MK919200.1) وسجلت 2 عزلات تنتمي الى *Ascaridia numidae* سجلت بالارقام (MK919207.1, MK919264.1). تعد الدراسة الاول في بغداد،العراق لتشخيص النوعين *Ascaridia numidae* و*Ascaridia nymphii* في الحمام الداجن باستخدام الطرق الجزيئية.

كلمات مفتاحية: سلسلة البلمرة، شجرة التطور الوراثي، مرض الاسكارس، الحمام، العراق

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INTRODUCTION

Birds may harbour numerous intestinal parasites, including protozoa, cestodes, trematodes and nematodes (8;19). Among parasites, the gastrointestinal helminthes *Ascaridia* ones may causing clinical signs(14). Ascaridiosis caused by *Ascaridia* is a most common nematoda worm in domestic birds. *Ascaridia* has a direct life cycle, The eggs of *Ascaridia* are resistant to the environment factors(22). Birds ingest infected eggs, directly with contaminated food and water or indirectly by transport host consumption (earthworm) (3). The egg hatches in the small intestine of their host. The released larvae can cause destruction and erosion of the intestinal mucosal layer and proliferation of mucus secreting cells (13). *Ascaridia* infections are often associated with reduced body condition, increased feed conversion ratio, and overall reduced health conditions (5).The infection may also act to depress the immune system of the host ,lead to increasing the severity of concurrent diseases (23). This study was conducted to detection an infection for *Ascarida* species in domestic pigeons fecal by microscopic examination and molecular assay.

MATERIALS AND METHODES

Samples collection

A total of 160 domestic pigeon (90 males and 70 females) were collected from 1/1/ 2019 to 31/3/2019. These birds were trapped alive from different areas of Baghdad city. All birds were transferred to Parasitology laboratory, college of Veterinary Medicine, University of Baghdad.Then, Fecal samples were removed from each pigeons into a dry petri dish from the cloacal orifice by gently squeezing the abdomen. Each fecal sample was divided into two parts the first one for the direct microscopic examination (direct wet smear) for the *Ascaridia* eggs identification were described according (21), the second part kept at -20 ^{o}C for molecular diagnosis by conventional PCR.

Microscopic examination

Direct smear were prepared and examined according to (4).

Molecular diagnosis Method

Genomic DNA extraction: Fifty fecal samples collected randomly from 160 feacal sample used in DNA extraction purpose according to commercial purification method described by G- spin DNA extraction kit cat.no. 17045 (INTRON biotechnology, Korya). A final DNA sample of 50 µl was eluted and stored at -20 ^{o}C until analyzed by PCR. Estimation of the DNA concentration and purity carried out according to a method presented by (6; 16). Red save stained 2% agarose gel applied for detected DNA quality and integrity (7; 10).

PCR reaction pair of oligonucleotide primer were used to perform a PCR by purified genomic DNA as a template against the *18s rRNA* gene of *Ascaridia* spp., primer designs by the (NCBI)gene-Bank data (Table 1).

Table1. The specific primer *Ascaridia* spp. of *18s rRNA* gene

Primer	Sequence	Tm (^{o}C)	GC (%)	Product size
Forward	5'- AGTGCTTAACGCGGGCTTAT - 3'	60.11	50.00	724
Reverse	5'- AAAGCACGCTGATTCCTCCA - 3'	59.96	50.00	base pair

PCR Thermo Cycler Conditions

PCR were done by using conventional PCR thermo cycler system as following(Table2).

Table2. The optimum conditions of detection *Ascaridia spp*

No.	Phase	Tm (^{o}C)	Time	No. of cycle
1-	Initial Denaturation	95^{o}C	3 min.	1
2-	Denaturation -2	95^{o}C	45sec	
3-	Annealing	60^{o}C	45sec	
4-	Extension-1	72^{o}C	45sec	35
5-	Extension -2	72^{o}C	10 min.	1

Sequencing and phylogenetic analysis

PCR products purified using (INTRON) kit and analyzer (Macrogen) using terminator cycle sequencing and BLAST analysis database (http://blast.ncbi.nlm.nih.gov), edited with (Mega 6) then analyses by (Neighbour Joining Method).

Statistical analysis: All data were Analysis by using Statistical System- SAS -2012) . The factors of differences were assessed by chi square test (18).

RESULTS AND DISCUSSION

Microscopic examination: The microscopic examination showed that *Ascaridia* eggs in feacal of infected pigeons figure (1).The result showed that the total infection rate of *Ascaridia* spp in fecal pigeons 15.62% (25/160) , which recorded in males 11.11% (10/90), while the females 21.42% (15/70),with significant difference ($P < 0.05$) (Table 3).

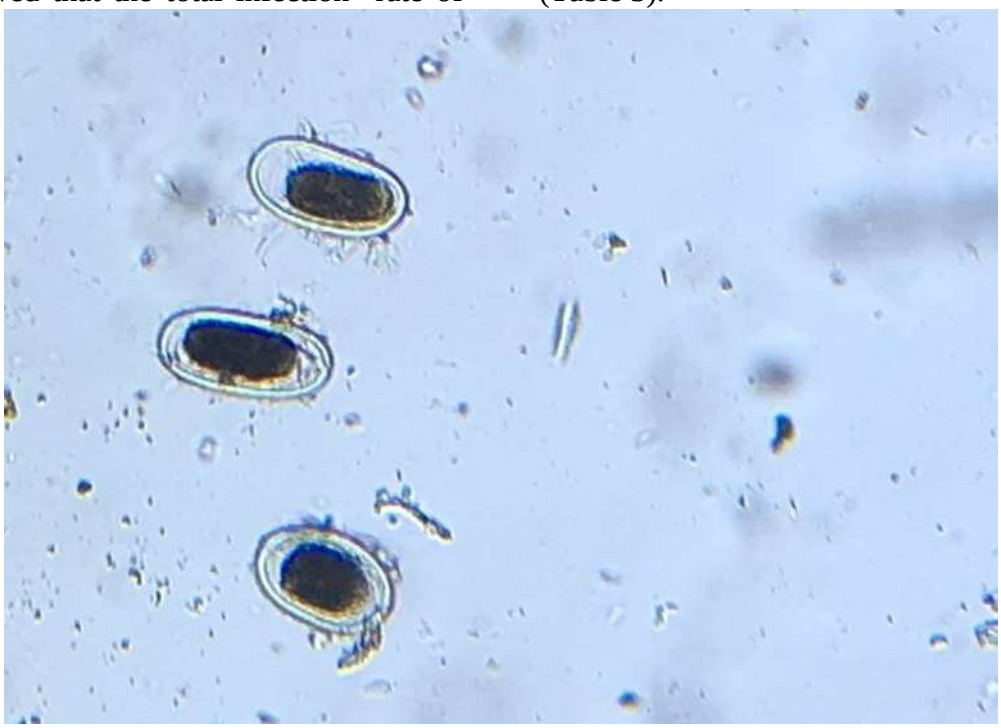

Figure1. Egg *of Ascaridia* spp. found in fecal samples of pigeons by direct wet smear(X10).

Table 3. Infection rate of *Ascaridia* spp, according to pigeon sexes

Sex	No. of animal examined	Infected	%
Males	**90**	**10**	**11.11**
Females	**70**	**15**	**21.42**
Total	**160**	**25**	**15.62**
Chi-Square (χ^2)	**---**	**---**	**4.362 ***
*** (P<0.05)**			

The current study was carried out to detection of *Ascaridia* spp. in domestic pigeons, prevalence rate (15.62%) of *Ascaridia* spp. were consistence with , 16.66%, 15.50% and 15.21% obtained by previous studies (11; 14; 15) respectively. The prevalence of present study was higher as compared to 5.10%, 4.04% and 1.20% of previous studies (2; 12;17) respectively. This is probably due to different climatic factors in the study areas (20). The high rate of infection in females . may be due to changes of hormonal, stress during laying of eggs , or female birds are more voracious in their feeding habits especially through the egg production than the males that remain largely selective (1).

Molecular diagnosis

Ascaridia species eggs are difficult to be differentiate for recognized microscopically, molecular assay have been used to detection and differentiate *Ascaridia* species (9).To the best of our knowledge, this is the first study to identify *Ascaridia* infection in domestic birds in Iraq using molecular techniques; it can therefore serve as a baseline for studies of *Ascaridia* in Iraq and Molecular techniques are more advantageous for *Ascaridia* identification as they more sensitive method. The PCR results revealed that eight samples found positive for *Ascaridia* from fifty(16%) pigeon feacal samples with an expected product size of 724 bp length (Figure 2).

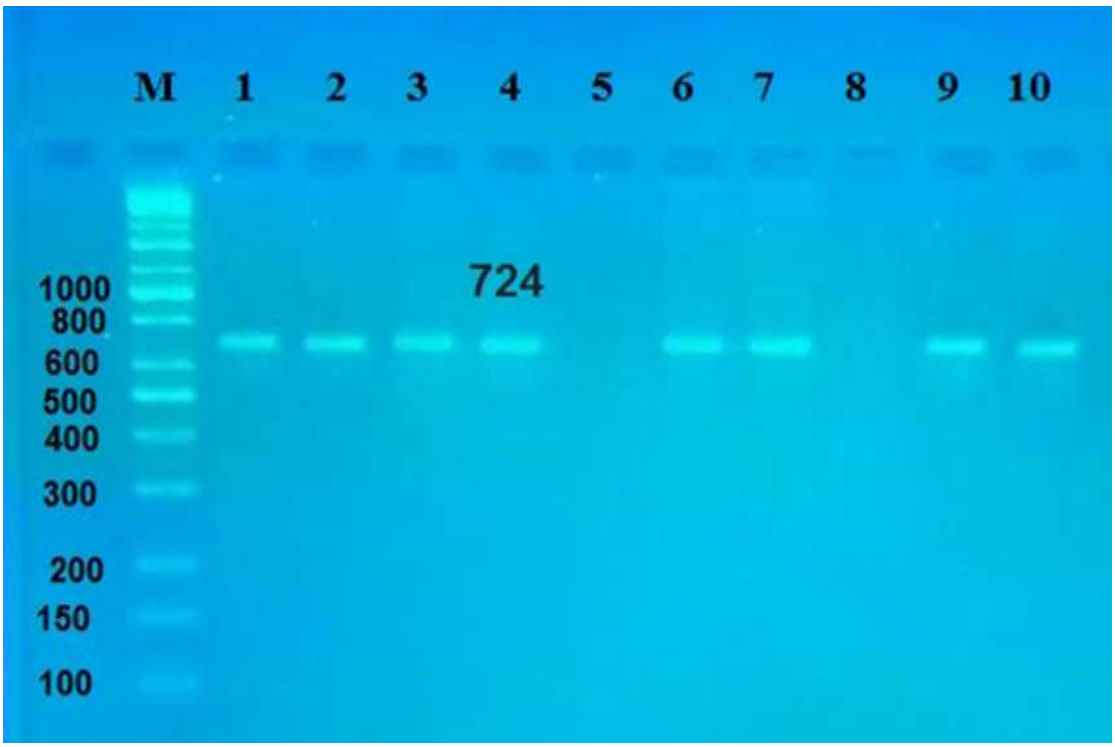

Figure 2. lines 1, 10: molecular marker (100bp); lines 1, 2, 3 , 4 6,7 , 9,10 positive for *Ascaridai* samples (724). lines 5,8 negative samples. The product was electrophoresis on 2% agarose at 5 volt/cm^2. . 1:30 hours.

In total, 8 PCR products were sequenced and deposited in Gene Bank in accession numbers; (MK918635.1, MK918636.1,MK918847.1, MK919081.1,MK919199.1, MK919200.1,MK919207.1, MK919264.1).

Phylogenetic analysis

The present study was the first study on *Ascaridia* spp that were isolated from pigeons in Iraq based on available information obtained from NCBI Gene Bank. The sequences have been registered in NCBI under accession numbers (MK918635.1, MK918636.1,MK918847.1,MK919081.1) belong to *Ascaridia galli*, (MK919199.1, MK919200.1)belong to *Ascaridia nymphii* and (MK919207.1, MK919264.1) belong to *Ascaridia numidae* for analysis and were compared with the NCBI- Gene bank *Ascaridia* strain isolates. Sequences result showed that the Iraqi isolate of *Ascaridia galli* was highly homology with (EF180058.1) from USA strain 99% , while the lowest homology with strain (KP982857.1)from Brazil strain96% , at total genetic changes (0.05-0. 35%), as shown in(Table4) (Figure 3).

Table 4.NCBI-BLAST homology sequence identity (%) between Iraqi isolate of *Ascaridia galli* and NCBI-BLAST *Ascaridia galli strain* isolate

	ACCESSION	Gene	Country	Source	Identity
1.	ID: EF180058.1	18S ribosomal RNA gene	USA	*Ascaridia galli*	99%
2.	ID: KP982857.1	18S ribosomal RNA gene	Brazil	*Ascaridia galli*	96%

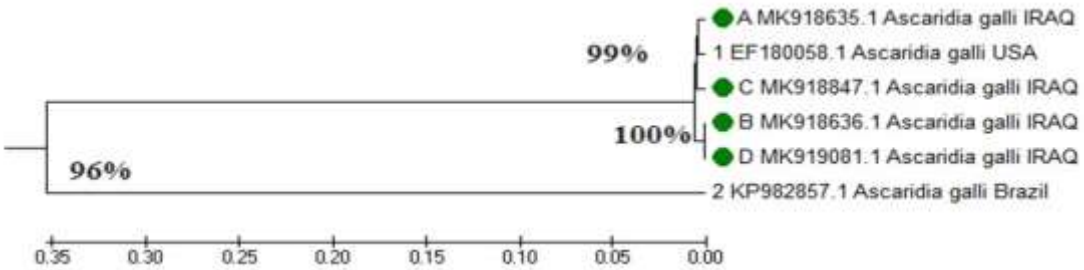

Figure 3. Phylogenetic tree analysis of *Ascaridia spp.* that used for country relationship analysis sequence. Green circle: *Ascaridia galli* isolated from Iraq

The Iraqi isolate of *Ascaridia nymphii* showed the highest homology with (MF375333.1, MF375332.1,MF375331.1, MF375330.1, MF375329.1)from China strain 99% , while the lowest homology (LC057210.1) isolate from Japan(Kanagawa) strain 98%, at total genetic changes (0.0005-0.0025%) (Table5) (Figure 4).

Table 5. NCBI-BLAST homology sequence identity (%) between Iraqi isolate of *Ascaridia nymphii* and NCBI-BLAST *Ascaridia nymphii strain* isolate

ACCESSION	Gene	Country	Source	Identity
3. ID: LC057210.1	18S ribosomal RNA gene	Japan:Kanagawa	*Ascaridia nymphii*	98%
4. ID: MF375333.1	18S ribosomal RNA gene	China	*Ascaridia nymphii*	99%
5. ID: MF375332.1	18S ribosomal RNA gene	China	*Ascaridia nymphii*	99%
6. ID: MF375331.1	18S ribosomal RNA gene	China	*Ascaridia nymphii*	99%
7. ID: MF375330.1	18S ribosomal RNA gene	China	*Ascaridia nymphii*	99%
8. ID: MF375329.1	18S ribosomal RNA gene	China	*Ascaridia nymphii*	99%

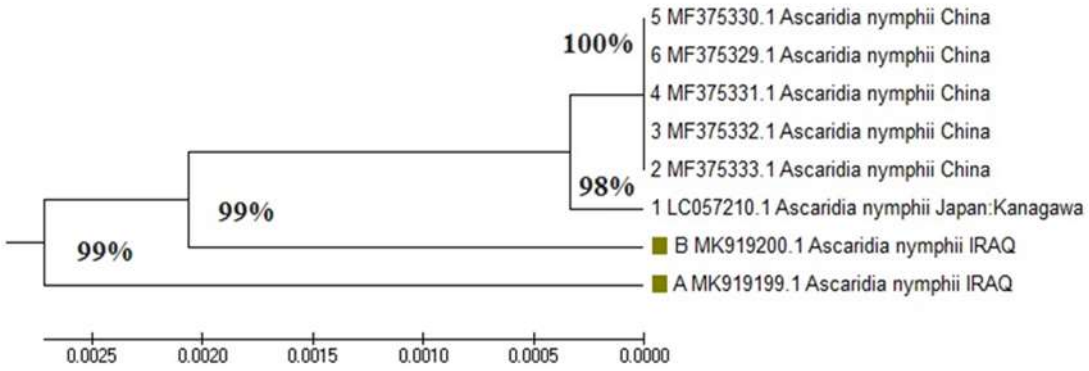

Figure 4. Phylogenetic tree analysis of *Ascaridia nymphii* that used for country relationship analysis. Yellow Square: *Ascaridia nymphii* isolated from Iraq

The Iraqi isolate of *Ascaria numidae* showed the highest homology with (HQ404166.1) from USA strain 99%, at total genetic changes (0.001-0.005%) (Table6) (Figure 5).

Table 6. NCBI-BLAST homology sequence identity (%) between Iraqi isolate of *Ascaridia numidae* and NCBI-BLAST *Ascaridia numidae strain* isolate

ACCESSION	Gene	Country	Source	Identity
9. ID: HQ404166.1	18S ribosomal RNA gene	USA	*Ascaridia numidae*	99%

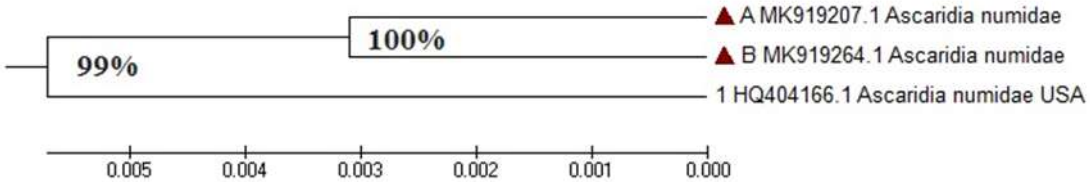

Figure 5. Phylogenetic tree analysis of *Ascaridia numidae* that used for country relationship analysis. Red triangle: *Ascaridia numidae* isolated from Iraq

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