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Original Article

Phylogenetical analysis of partially sequenced *cytb* gene of *Haemoproteus columbae* in pigeons and its pathological lesions in Egypt

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Abstract

Background: *Haemoproteus columbae* is widely distributed in tropical and subtropical regions, causing pseudomalaria in pigeons. **Aims:** The current study aimed to characterize the phylogenetic position of *H. columbae* in pigeons in Sharkia province, Egypt, based on partial sequencing of the *cytb* gene as the conserved regions. The "DNA barcode" of the *cytb* gene helps in designing primers that can be used to amplify the same gene in the related haemosporidians. **Methods:** One hundered blood samples were collected from domestic pigeons to identify *H. columbae* by polymerase chain reaction (PCR) and detect its relationship with other related haemosporidians. **Results:** Weight losses of 60%, anemia 40%, low growth rates 26.67%, diarrhea 76.67%, dyspnea 66.67%, some neurological symptoms 33.33%, and death 16.67% were observed in the studied birds. Post-mortem examinations showed chocolate-brown appearance of the livers of the birds and congested parenchymatous organs. Microscopical examinations of Giemsa stained blood smears (n=100) revealed a 30% infection rate. The obtained infection percentages were more pronounced in males (35.71%) than females (16.66%) and more in adults (57.14%) than young pigeons (15.38%). The present sequence of *H. columbae* was deposited in GenBank under accession No.: MH345964 and shows 100% identity with other related *Haemoproteus* species in the Sao Paulo Zoo, Brazil (KU131585 and KU131583) and the UK (KX832581 and KX832586). **Conclusion:** This study concluded that the accurate diagnosis of *H. coulmbae* infection in pigeons by specific primers will help with the early treatment of affected cases, especially in the presence of the immature forms, and can thus avoid the noticed clinical signs and the induced pathological lesions mentioned in our study.

Key words: Avian, Disease, Infection, *Haemoproteus columbae*, Pigeon

Introduction

Pigeons are considered to be potential carriers of zoonotic parasites because of their interactions with man and other domestic and wild birds. *Haemoproteus columbae* infection is widely distributed in tropical and subtropical regions (Springer, 1972), causing pseudomalaria or pigeon malaria which is fatal to young pigeons (Borkataki *et al*., 2015). Infected birds show loss of body weight, dullness, depression, dyspnoea, torticollis, diarrhea, anemia, anorexia, dehydration, and finally death (Nematollahi *et al*., 2012; Joshi *et al*., 2017). *Haemoproteus columbae* has subclinical pathogenic effects except in acute forms of infection, where heavy mortality has been recorded (Dey *et al*., 2010). In addition, previously infected pigeons become immunized against re-infection with *H. columbae* (Ahmed and Mohammed, 1978).

Haemoproteus columbae was studied for the first time by Kruse 1890 in *Columba livia* pigeons' blood. *Haemoproteus* spp. are intracellular parasites transmitted by blood-sucking insects, including mosquitoes, biting midges, *Pseudolynchia canariensis* and tabanid flies (Al-Barwari and Saeed, 2012). Once the vector bites birds, the sporozoites are released in the bloodstream, invade endothelial cells of blood vessels of the lung, liver and spleen, and produce schizonts, which in turn produce numerous merozoites. They later penetrate red blood cells (RBCs) and transform into gametes. Then, the insect vector takes blood from infected birds and undergoes sexual reproduction producing oocysts. The oocysts rupture and release several sporozoites that invade the salivary gland and act as a focus for subsequent infection (Soulsby, 1982; Rupiper, 1998; Taylor *et al*., 2007).

The reproduction of *Columba livia* pigeons is of economic importance to Egypt as domestic pigeons, especially the younger ones (squabs), are mainly bred for their meat (Hussein and Abdelrahim, 2016). Therefore, this study was conducted to identify the clinical symptoms and pathological lesions caused by *H. columbae* in infected pigeons. In Egypt, domestic pigeons are considered a member of the poultry family. The population of raised pigeons reached more than

2,500,000 in 2003 (Lane, 2003) and later on about 11,300,000 in 2018 according to FAO. The early and accurate diagnosis of *H. columbae* would thus help with the effective treatment of infected pigeons in these populations.

The ability of *H. columbae* to infect domestic pigeon populations and risk their health has caused an economically marked decrease in reproductive success. Due to the pigeons' role in transmitting infection with this parasite, and its significant impact on the overall status of the bird population, the current study aimed to determine the prevalence, describe pathological lesions in visceral organs and find the molecular characteristics and phylogenetic positions of *H. columbae* in pigeons in the Sharkia province, Egypt, based on partial sequencing of the *cytb* gene.

Materials and Methods

Bird collection and sampling

A total of 100 domestic pigeons were examined. These cases belonged to different localities admitted to the Veterinary Clinic, Faculty of Veterinary Medicine, Zagazig University, Sharkia province, Egypt. The examined pigeons included 70 males and 30 females, and their ages ranged between 2 months to 3 years. The blood samples were collected in tubes containing Ethylenediaminetetraacetic acid (EDTA) from the pigeons' wing vein. A small drop of the blood (EDTA) sample was placed on a clean glass slide. A thin blood smear was then prepared and allowed to be air-dried and fixed with absolute methyl for 10 min. The sample was then stained with freshly prepared Giemsa stain and examined under an oil immersion lens to identify and differentiate macro and micro gametocytes of *H. columbae* according to the characters described by (Soulsby, 1982). The remained blood samples were kept at -20°C until DNA extraction.

This study was ethically approved by ZU-IACUC Committee, Zagazig University, Egypt (ZU-IACUC/2/F/6/2018).

Histopathology

The collected specimens from the liver, lung, and heart were fixed in a 10% buffered formalin solution, dehydrated in gradual alcohol (70-100%), cleared in xylene and embedded in paraffin. Five-micron thickness paraffin sections were prepared and stained with haematoxylin and eosin (H&E) dyes and examined microscopically (Suvarna *et al*., 2013).

Polymerase chain reaction (PCR) assay, sequencing, and phylogenetic analysis

DNA extraction from blood samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Amplification of the extracted DNA required a reaction mixture (25 µL) including 1.5 µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 0.25 µL of 20 pmol of each primer (*H. clom-F* 5´-TTA GAT ACA TGC ATG CAA CTG GTG-3´ and *H. clom-R* 5´-TAG TAA TAA CAG TTG CAC CCC AG-3´, Bio Basic Canada Inc.), 5 µL of undiluted DNA and up to 25 µL nuclease-free water. The PCR cycling program included an initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 5 min (Doosti *et al*., 2014).

The PCR products were purified using QIAquick PCR product extraction kit. (Qiagen, Valencia), sequenced using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) in an Applied Biosystems3130 genetic analyzer (HITACHI, Japan). The obtained sequences were aligned and compared with other associating sequences in the GenBank database by Basic Local Alignment Search Tool (BLAST®) tool (Altschul *et al*., 1990). The phylogenetic tree was created by the MegAlign module of Lasergene DNAStar (Thompson *et al*., 1994) and the phylogenetic analyses were performed using maximum likelihood in MEGA6 (Tamura *et al*., 2013). Sequence identity percentages were calculated using pairwise comparisons of the aligned sequence data.

Results

Clinical findings and PM lesions

The ante mortem (AM) examination of some pigeons revealed weight losses of 60%, anemia 40%, low growth rates 26.67%, diarrhea 76.67%, dyspnea 66.67%, and death 16.67% (Table 1). Neurological symptoms 33.33%

Table 1: Infection percentages and noticed symptoms of examined pigeons according to age and sex

Item	Ex. No.	Infect. % (No.)	Noticed symptoms % (Noticed No./Infected No.)						
			Weight losses	Anemia	Low growth	Diarrhea	Dyspnea	Neuro.	Death
Male	70	35.71% (25)	60% (15/25)	40% (10/25)	32% (8/25)	80% (20/25)	60% (15/25)	24% (6/25)	16% (4/25)
Female	30	16.66% (5)	60% (3/5)	40% (2/5)	0% (0/5)	60% (3/5)	100% (5/5)	80% (4/5)	20% (1/5)
Adult (7 months-3 years)	35	57.14% (20)	80% (16/20)	35% (7/20)	15% (3/20)	90% (18/20)	55% (11/20)	25% (5/20)	15% (3/20)
Young (2-6 months)	65	15.38% (10)	20% (2/10)	50% (5/10)	50% (5/10)	50% (5/10)	90% (9/10)	50% (5/10)	20% (2/10)
Total	100	30% (30)	60% (18/30)	40% (12/30)	26.67% (8/30)	76.67% (23/30)	66.67% (20/30)	33.33% (10/30)	16.67% (5/30)

Ex.: Examined, Infect.: Infection, Neuro.: Neurological symptoms, and No.: Number

were also recorded including movement of the head in circular or backward directions and torticollis episodes. Post-mortem examinations showed a chocolate-brown appearance of the liver with a septicemic picture (congestion of the parenchymatous organs) (Figs. 1A to C).

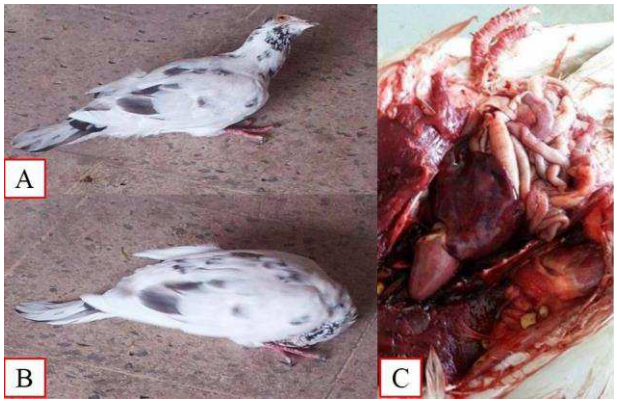

Fig. 1: Clinical signs and post mortem lesion of pigeons suspected to be infected with *Haemoproteus columbae*. (**A**) Backward direction of pigeon's head, (**B**) Torticollis, and (**C**) Chocolate brown appearance of the liver in the 6-month-old pigeon

Morphological identification

Infection percentages were found by microscopical examinations of Giemsa stained thin blood smears from 100 pigeons (Table 1).

Three forms of *H. columbae* blood stages were described as the following:

Immature forms

These forms reached 2.8-7.5 µm in length and 2.5-3.2 µm in width. They were situated lateral to the cell nucleus and not attached to the host cell membrane (Figs. 2A and B).

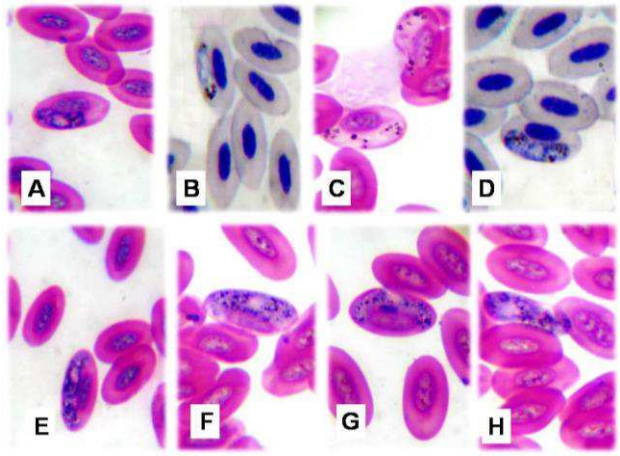

Fig. 2: Microphotographs of *Haemoproteus columbae* (×1000). (**A**) Immature macrogametocyte, (**B**) Immature micro-gametocyte, (**C**) Mature microgametocyte broad at one end and narrow at the other, (**D**) Mature microgametocyte rounded at both poles, (**E**) Mature macrogametocyte encircled host cell nucleus, (**F&G**) Mature macrogametocyte displaced host cell nucleus to periphery, and (**H**) Extra-corpuscular form

Mature forms

These forms were partially encircled and might displace the host cell nucleus. Their ends might be curved at both poles or broad at one pole and narrow at the other. They included microgametocytes and macrogametocytes. Microgametocytes measured 8.1-11.3 µm long and 1.2-3 µm wide. Their granules were regularly arranged at both poles (Figs. 2C and D). Macrometocytes measured 12-14.2 µm long and 2.1-5.8 µm wide. The granules were irregularly scattered and colored brown to black (Figs. 2E-G).

Extra-corpuscular forms

These forms appeared elongated outside RBCs with a granular cytoplasm and dispersed granules. They measured 5.5-15 µm in length and 1.5 to 8 µm in width (Fig. 2H).

Histopathological changes

The liver exhibited slight congestion of the portal vein with thickening, hyalinization of tunica media, and perivascular edema beside few mononuclear cells infiltration (Fig. 3A). Vacuolar degeneration was noticed in hepatocytes with aninterstitial aggregation of round cells and fibroblasts (Fig. 3B). Round shaped thin-walled megaloschizonts of *H. columbae* were observed inside the hepatic parenchyma (Fig. 3C). Hydropic degeneration of hepatic cells and congestion of central vein with a perivascular aggregation of mononuclear inflammatory cells were also observed (Fig. 3D). The lungs revealed the presence of *H. columbae* schizonts within the pulmonary blood vessel which was dilated and

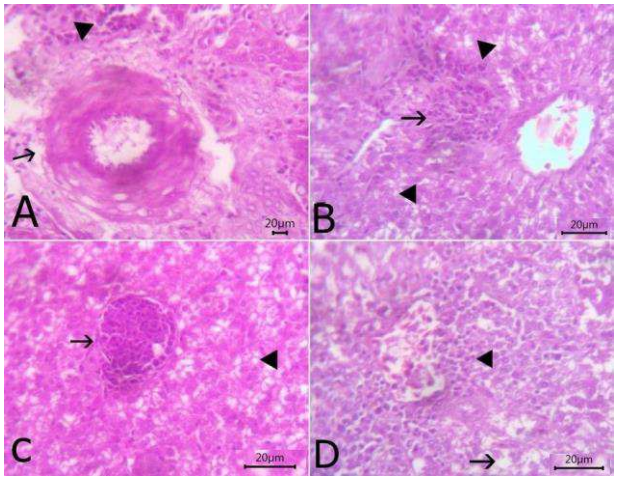

Fig. 3: The histopathological lesions in liver sections stained with H&E. (**A**) The liver showing slight congestion of portal vein with thickening of the wall and perivascular edema (arrow) beside few mononuclear cells infiltration (arrowhead) (bare=20), (**B**) Liver showing vacuolar degeneration of hepatocytes (arrowhead) with interstitial aggregation of round cells and fibroblasts (arrow) (bare=20), (**C**) Liver showing round-shaped thin-walled megaloschizont of *Haemoproteus columbae* (arrow) and vacuolation of hepatocytes (arrowhead) (bare=20), and (**D**) Liver showing hydropic degeneration of hepatic cells (arrow) and congestion of central vein with a perivascular aggregation of mononuclear inflammatory cells (arrowhead) (bare=20)

congested with thickened interalveolar septa due to the presence of extravasated erythrocytes (Figs. 4A and B). Schizonts were also present inside the alveoli, destroying its wall with emphysematous alveoli. Perivascular edema was detected around congested blood vessels (Fig. 4C). Catarrhal bronchitis was observed, characterized by metaplasia of lining epithelium into goblet cells with the desquamation of lining epithelium and leukocytic infiltration (Fig. 4D). There was round cell granuloma, which consisted of a central area of caseous necrosis surround by mononuclear inflammatory cells (Fig. 4E). The heart showed extravasated erythrocytes among the cardiac muscles beside the degeneration of the myocardium (Fig. 4F). Congestion of blood vessels with hyaline thickening of walls and perivascular edema were also observed (Fig. 4G).

Molecular characterization (PCR assay), sequence polymorphism, and phylogenetic analysis

For PCR, the target sequence chosen for amplification was part of the mitochondrial *cytb* gene. Those variable regions had shown to be suitable genetic markers for distinguishing *H. columbae.* The length of their PCR products was 204 bp. The BlAST search results for *H. columbae* (accession No.: MH345964) in the current study revealed 100% identity with KU131585 and KU131583 in the Sao Paulo Zoo, Brazil, KX832581 and KX832586 in the UK, respectively. These sequences shared the same clade but the identity percentage reached 92.2% with *Haemoproteus multipigmentatus* (JN788943), 91.7% with *Haemoproteus antigonis* (KX223844) and 91.2% with *Plasmodium relictum* (HM031936). The phylogenetic tree based on *cytb* gene sequences showed that the currently studied *H. columbae* (MH345964) shared the same clade with *H. multipigmentatus*, while, *H. antigonis* and *Plasmodium relictum* had a separate clade (Figs. 5 and 6).

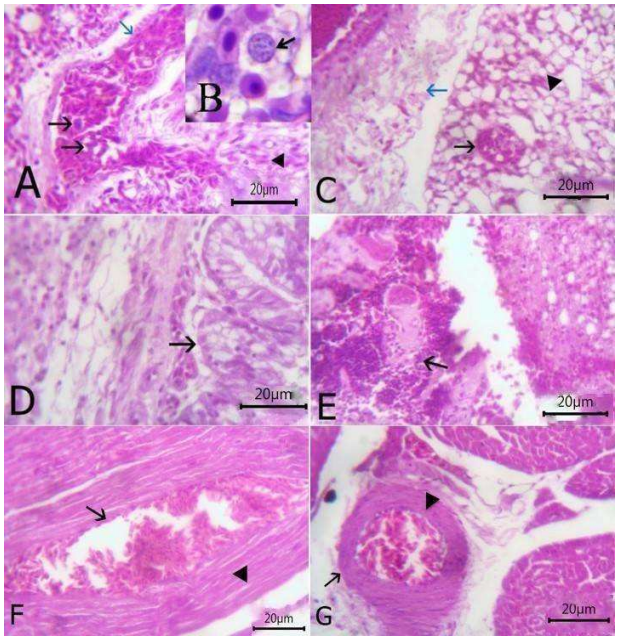

Fig. 4: (**A**) Lung section stained with H&E showing the presence of *Haemoproteus columbae* schizont within pulmonary blood vessel (arrow), dilated and congested (blue arrow) with presence extravasated erythrocytes (arrowhead) (bare=20), (**B**) Higher magnification of schizont (arrow), (**C**) Lung showing schizont inside the alveoli (arrow) with some emphysematous alveoli (arrowhead) and perivascular edema around congested blood vessels (blue arrow) (bare=20), (**D**) Lung showing catarrhal bronchitis (arrow) (bare=20), (**E**) Lung showing granuloma which consisting of the central area of caseous necrosis surrounded by mononuclear inflammatory cells (arrow) (bare=20), (**F**) Heart showing extravasated erythrocytes (arrow) among degenerated cardiac muscles (arrowhead) (bare=20), and (**G**) Heart showing congestion of blood vessels with hyaline thickening of its wall (arrow) and perivascular edema (arrowhead) (bare=20)

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
1		100.0	100.0	100.0	99.5	100.0	99.5	99.0	99.0	99.0	98.5	98.0	98.0	99.0	92.2	92.2	92.6	92.2	91.7	91.7	91.7	90.7	91.7	91.2	90.7	90.7	90.7	1
2	0.0		100.0	100.0	99.5	100.0	99.5	99.0	99.0	99.0	98.5	98.0	98.0	99.0	92.2	92.2	92.6	92.2	91.7	91.7	91.7	90.7	91.7	91.2	90.7	90.7	90.7	2
3	0.0	0.0		100.0	99.5	100.0	99.5	99.0	99.0	99.0	98.5	98.0	98.0	99.0	92.2	92.2	92.6	92.2	91.7	91.7	91.7	90.7	91.7	91.2	90.7	90.7	90.7	3
4	0.0	0.0	0.0		99.5	100.0	99.5	99.0	99.0	99.0	98.5	98.0	98.0	99.0	92.2	92.2	92.6	92.2	91.7	91.7	91.7	90.7	91.7	91.2	90.7	90.7	90.7	4
5	0.5	0.5	0.5	0.5		99.5	99.0	98.5	98.5	98.5	98.0	97.5	97.5	98.5	92.6	92.6	93.1	92.6	91.2	91.2	91.2	90.2	92.2	90.7	90.2	90.2	90.2	5
6	0.0	0.0	0.0	0.0	0.5		99.5	99.0	99.0	99.0	98.5	98.0	98.0	99.0	92.2	92.2	92.6	92.2	91.7	91.7	91.7	90.7	91.7	91.2	90.7	90.7	90.7	6
7	0.5	0.5	0.5	0.5	1.0	0.5		98.5	98.5	98.5	98.0	97.5	97.5	98.5	91.7	91.7	92.2	91.7	91.2	91.2	91.2	90.2	91.2	90.7	90.2	90.2	90.2	7
8	1.0	1.0	1.0	1.0	1.5	1.0	1.5		98.0	98.0	97.5	97.1	97.1	98.0	91.2	91.2	91.7	91.2	91.7	91.7	91.7	90.7	90.7	90.7	90.2	90.7	90.7	8
9	1.0	1.0	1.0	1.0	1.5	1.0	1.5	2.0		100.0	99.5	99.0	99.0	100.0	91.2	91.2	91.7	91.2	91.7	91.7	91.7	90.7	92.6	91.2	90.7	89.7	89.7	9
10	1.0	1.0	1.0	1.0	1.5	1.0	1.5	2.0	0.0		99.5	99.0	99.0	100.0	91.2	91.2	91.7	91.2	91.7	91.7	91.7	90.7	92.6	91.2	90.7	89.7	89.7	10
11	1.5	1.5	1.5	1.5	2.0	1.5	2.0	2.5	0.5	0.5		99.0	99.0	99.5	91.2	91.2	91.7	91.2	91.7	91.7	91.7	90.7	92.6	91.2	91.2	89.7	89.7	11
12	2.0	2.0	2.0	2.0	2.5	2.0	2.5	3.0	1.0	1.0	1.0		100.0	99.0	91.2	91.2	91.7	91.2	91.2	91.2	91.2	90.2	92.6	91.2	90.2	89.7	89.7	12
13	2.0	2.0	2.0	2.0	2.5	2.0	2.5	3.0	1.0	1.0	1.0	0.0		99.0	91.2	91.2	91.7	91.2	91.2	91.2	91.2	90.2	92.6	91.2	90.2	89.7	89.7	13
14	1.0	1.0	1.0	1.0	1.5	1.0	1.5	2.0	0.0	0.0	0.5	1.0	1.0		91.2	91.2	91.7	91.2	91.7	91.7	91.7	90.7	92.6	91.2	90.7	89.7	89.7	14
15	8.3	8.3	8.3	8.3	7.7	8.3	8.8	9.4	9.4	9.4	9.4	9.4	9.4	9.4		98.0	98.5	99.0	88.7	88.7	88.7	87.7	97.5	86.8	85.8	89.2	89.2	15
16	8.3	8.3	8.3	8.3	7.7	8.3	8.8	9.4	9.4	9.4	9.4	9.4	9.4	9.4	2.0		97.5	97.1	88.2	88.2	88.2	87.3	98.5	86.8	86.8	88.7	88.7	16
17	7.7	7.7	7.7	7.7	7.2	7.7	8.3	8.8	8.8	8.8	8.8	8.8	8.8	8.8	1.5	2.5		99.5	88.2	88.2	88.2	87.3	97.1	87.3	86.3	88.7	88.7	17
18	8.3	8.3	8.3	8.3	7.7	8.3	8.8	9.4	9.4	9.4	9.4	9.4	9.4	9.4	1.0	3.0	0.5		88.2	88.2	88.2	87.3	96.6	86.8	85.8	88.7	88.7	18
19	8.8	8.8	8.8	8.8	9.4	8.8	9.4	8.8	8.8	8.8	8.8	9.4	9.4	8.8	12.3	12.9	12.8	12.9		100.0	100.0	99.0	88.7	90.7	91.7	93.1	93.1	19
20	8.8	8.8	8.8	8.8	9.4	8.8	9.4	8.8	8.8	8.8	8.8	9.4	9.4	8.8	12.3	12.9	12.8	12.9	0.0		100.0	99.0	88.7	90.7	91.7	93.1	93.1	20
21	8.8	8.8	8.8	8.8	9.4	8.8	9.4	8.8	8.8	8.8	8.8	9.4	9.4	8.8	12.3	12.9	12.8	12.9	0.0	0.0		99.0	88.7	90.7	91.7	93.1	93.1	21
22	9.9	9.9	9.9	9.9	10.5	9.9	10.5	9.9	10.0	10.0	10.0	10.5	10.5	10.0	13.5	14.0	14.0	14.0	1.0	1.0	1.0		87.7	90.2	91.7	92.2	92.2	22
23	8.8	8.8	8.8	8.8	8.3	8.8	9.4	9.9	7.7	7.7	7.7	7.7	7.7	7.7	2.5	1.5	3.0	3.5	12.3	12.3	12.3	13.5		87.3	87.3	88.2	88.2	23
24	9.4	9.4	9.4	9.4	10.0	9.4	9.9	10.0	9.4	9.4	9.4	9.4	9.4	9.4	14.6	14.6	14.0	14.6	10.0	10.0	10.0	10.5	14.0		94.1	92.6	92.6	24
25	9.9	9.9	9.9	9.9	10.5	9.9	10.5	10.5	10.0	10.0	9.4	10.5	10.5	10.0	15.8	14.6	15.2	15.8	8.8	8.8	8.8	8.8	14.0	6.1		90.7	90.7	25
26	9.9	9.9	9.9	9.9	10.5	9.9	10.5	9.9	11.1	11.1	11.1	11.1	11.1	11.1	11.7	12.2	12.2	12.2	7.2	7.2	7.2	8.3	12.8	7.8	10.0		100.0	26
27	9.9	9.9	9.9	9.9	10.5	9.9	10.5	9.9	11.1	11.1	11.1	11.1	11.1	11.1	11.7	12.2	12.2	12.2	7.2	7.2	7.2	8.3	12.8	7.8	10.0	0.0		27
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	

KX832586 Haemoproteus sp.
Egy strain MH345964
KU131585 Haemoproteus columbae
KU131583 Haemoproteus columbae
KT290924 Haemoproteus sp.
KX832581 Haemoproteus sp.
KT290922 Haemoproteus sp.
KX832584 Haemoproteus sp.
KF537314 Haemoproteus columbae
EU254548 Haemoproteus columbae
KY653761 Haemoproteus columbae
FJ168562 Haemoproteus columbae
EU254553 Haemoproteus columbae
KJ488803 Haemoproteus sp.
JN788943 Haemoproteus multipigmentatus
JN788936 Haemoproteus multipigmentatus
JN788942 Haemoproteus multipigmentatus
JN788947 Haemoproteus multipigmentatus
KX223844 Haemoproteus antigonis
KX223843 Haemoproteus antigonis
KX223841 Haemoproteus antigonis
KX223842 Haemoproteus antigonis
FJ462670 Haemoproteus sp.
HM031936 Plasmodium relictum
MG018694 Plasmodium sp.
KX604232 Plasmodium sp.
KU296187 Plasmodium sp.

Fig. 5: A sequence identity matrix of *cytb* gene (upper right triangle), reconstructed from protein sequences and showing the sequence distance (lower left triangle) for the Egy strain of *Haemoproteus columbae* (MH345964) with other related haemosporidian parasites in birds

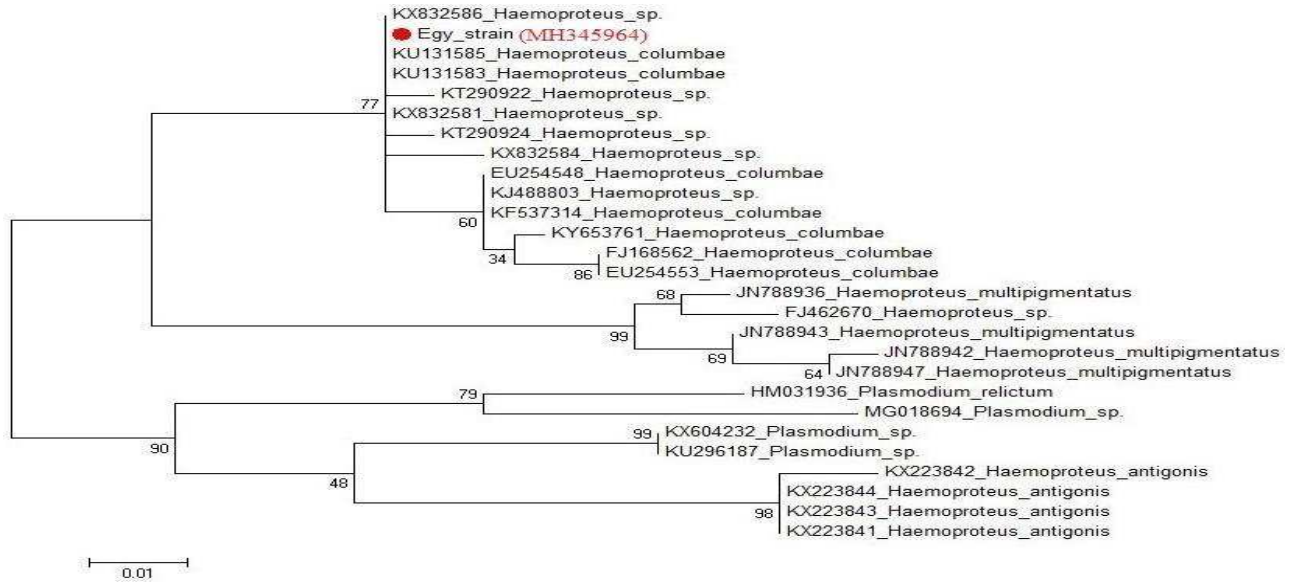

Fig. 6: Phylogenetic tree showing genetic relationship between *cytb* gene sequences of *Haemoproteus columbae* in this study (marked with red circle) and other sequences in the GenBank. The tree was generated maximum likelihood in MEGA6

Discussion

Infection with *H. columbae* is known as pseudomalaria because of its similarity with *Plasmodium* species (Friend and Franson, 1999). Most infections with *H. columbae* in pigeons were asymptomatic. However, young and immunocompromised pigeons might suffer from the disease. The clinical examination of pigeons in the present study revealed anorexia, depression, inability to fly, circling movements and episodes of torticollis. Postmortem examinations revealed livers with chocolate brown appearances and congested parenchymatous organs. These were similar to the observations recorded by (Varshney *et al*., 2014; Maharana and Kumar, 2017; Ortiz-Catedral *et al*., 2019) and the noticed *in vivo* gametocytes recorded by Coral *et al*. (2015).

Diagnosis of infected birds with *H. columbae* depended mainly upon the microscopical examination of Giemsa-stained blood smears. Gametocytes were only detected in RBCs. They are characterized by multiple, refractile, and golden brown pigment granules (haemozoin) arose from haemoglobin digestion (Friend and Franson, 1999). Gametocytes also appeared halter-shaped and some encircled the host cell nucleus, inducing cell distortion and nuclear displacement (Samani *et al*., 2013; Hussein and Abdelrahim, 2016). Only one gametocyte was detected inside the infected red cells of pigeons. This is considered as an indicator of mild infection, according to (Gicik and Arslan, 2001; Samani *et al*., 2013; Borkataki *et al*., 2015).

By examining microscopical Giemsa staining films, we recorded a 30% infection percentage (total) with *H. columbae* in pigeons in Sharkia province, Egypt. Similar rates were reported to be 26.7% by Martinez-Moreno *et al*. (1989) in Spain, 33% by Razmi and Andalibian (2006) in the Northeast of Iran, 37% by Msoffe *et al*. (2010) in Tanzania, 30% by Youssefi and Rahimi (2011) in Iran, 29.47% by Abed *et al*. (2014) in Iraq, and 29.4% by Scaglione *et al*. (2015) in Italy.

Higher rates were reported to be 43.2% by Gulander *et al*. (2002) in Turkey, 43.63% by Islam *et al*. (2014) in Chittagong district, Bangladesh, 50% by Borji *et al*. (2011 and 2012) in Iran, 55.63% by Gupta *et al*. (2011) in India, 57% in Ankara by Gicik and Arslan (2001), 57.2% in Qena, Egypt by Hussein and Abdelrahim (2016), 58.25% by Islam *et al*. (2014) Khulna district, Bangladesh, 61.33% by Borkataki *et al*. (2015) in Jammu district, India, 62% by Nematollahi *et al*. (2012) in Isfahan, Iran, 73% by Earle and Little (1993) in South Africa, 74% by Yunus and Arsalan (2001) in Turkey, 75% by Mushi *et al*. (1999) in Botswana, 76.5% by Dranzoa *et al*. (1999) in Uganda, and 80% in Sebele by Mushi *et al*. (2000).

Lower rates were reported to be 14% in Lapai, Nigeria by (Dadi-Mamud *et al*., 2012), 15.6% in Nigeria (Natala *et al*., 2009), 17.47% in North Iran (Youssefi *et al*., 2010), 18.8% in Turkey (Senlik *et al*., 2005), 22.3% in Bangladesh (Zahan *et al*., 2018), 22.7% in Tangail, Bangladesh (Abdul Momin *et al*., 2014), 23.18% in Iran (Doosti *et al*., 2014), and 24% in Southwest of Iran (Samani *et al*., 2013).

Variations in infection rates might have resulted from different geographic distribution, bird habitat and physiological differences, feeding habits, housing systems, abundance of insect vectors, and proper usage of insecticides against pigeon fly larvae in crevices of pigeon houses or in their nests.

Contrary to several studies (Earle and Little, 1993; Samani *et al*., 2013; Abed *et al*., 2014; Saikia *et al*., 2019), our obtained infection percentage was higher in male compared to female pigeons. Nevertheless, our findings matched those of Al-Barwari and Saeed, (2012), Hussein and Abdelrahim, (2016), and Zahan *et al*. (2018). Concerning the age factor, the obtained infection rate was more pronounced in adults than younger ages, and this was similar to Samani *et al*. (2013) and Zahan *et*

al. (2018). On the other hand, Senlik *et al*. (2005) and Scaglione *et al*. (2015) could not detect a significant relationship between age or sex and infection rates. According to Jones (2006), not only environmental and genetic factors affect the resistance of birds to blood parasites, but age and sex of the birds, parasite strain, and stress also induce different infection rates. We also suggest that males were more susceptible to infection possibly due to their:

(a) Differences in appearance with females

(b) Mating habits, whereby each male mates with several females which might be infested with pigeon fly

(c) Testosterone which has an immunosuppressive role which hinders the clearance of the parasitic infection

Histopathological changes were exhibited within examined organs, including erosion of the blood vessels with lysis of their endothelial lining during the development of *H. columbae* schizonts in blood vessels of infected pigeons. In such a case, they infect another host they are directed towards through the endothelial cells of the blood vessels (Cottin *et al*., 1998). The liver revealed congestion of the portal vein with thickening, and the hyalinization of tunica media beside few mononuclear cell infiltrations. Vacuolar degeneration was noticed in hepatocytes with an interstitial aggregation of round cells. Round shaped thin-walled megaloschizonts of *H. columbae* were observed inside the hepatic parenchyma. These findings partially agreed with Hussein and Abdelrahim (2016) and Cepeda *et al*. (2019), who observed moderate and multifocal lymphoplasmocytic hepatitis with hepatocyte degeneration and vacuolated cytoplasm. In addition, *H. columbae* meronts were present inside the hepatic sinusoid. Microscopic changes in the lung showed *H. columbae* schizonts within the pulmonary blood vessel and inside the alveoli, destroying its wall with emphysematous alveoli. Round cell granuloma was observed, which consisted of the central area of the caseous necrosis surrounded by mononuclear inflammatory cells. These results partially agreed with those of Mubarak and Abed (2005) who reported that the pulmonary tissue was the main target for *H. columbae* schizonts in pulmonary blood vessels, and caused granulomatous pulmonary tissue reactions with emphysematous alveoli and other collapsed alveoli. The heart showed extravasated erythrocytes among cardiac muscles beside the degeneration of myocardium. These findings agreed with Olias *et al*. (2011) who observed necrosis and haemorrahge inside heart muscle fibers.

Recently, the *cytb* gene has been extensively used as a suitable target for the accurate identification of *H. columbae*. The conserved regions of the *cytb* gene "DNA barcode" help to design primers that can be used to amplify the same gene in the related species (Bensch *et al*., 2009). Many authors used PCR to identify and differentiate *H. columbae* affecting pigeons. In our study, the length of the PCR products was found to be 204 bp. This result was similar to those obtained by previous studies (Doosti *et al*., 2014; Maharana and Kumar, 2017; Saikia *et al*., 2019). The primers were also specifically used for the detection of *H. columba* in pigeons as previously described (Doosti *et al*., 2014; Maharana and Kumar, 2017; Saikia *et al*., 2019). On the other hand, the primers used by Hellgren *et al*. (2004) or Pacheco *et al*. (2011) could not specify the genus and species of the Haemosporidian infection (either *Plasmodium* sp. or *Haemoproteus* sp.).

Sequence analysis for the *cytb* gene revealed that the Egy strain of *H. columbae* (accession No.: MH345964) in domestic pigeons in Sharkia province, Egypt, shared 100% similarity with others in Brazil and the UK. Besides, the phylogenetic analysis in our study showed that *H. columbae* and *H. multipigmentatus* shared the same clade as those reported by Chagas *et al*. (2016) in São Paulo Zoo, Brazil.

Conflict of interest

The authors declare that there is no conflict of interest.

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