Prevalence and Molecular Detection of Blood Protozoa in Domestic Pigeon

Munmi Saikia1*, Kanta Bhattacharjee1, Prabhat Chandra Sarmah1, Dilip Kr. Deka1, Shantanu Tamuly2, Parikshit Kakati1 and Pranab Konch3

1Department of Parasitology, 2Department of Biochemistry, 3Department of Pathology, College of Veterinary Science, Khanapara, Guwahati-781022, Assam, India

*Corresponding author

ABSTRACT

The present study was carried out to know the status of haemoprotozoan infection of domestic pigeon in Assam by microscopic examination of blood of pigeons for a period of one year which revealed an overall prevalence of 53.39%. Three species viz. Haemoproteus columbae (29.93%), Plasmodium relictum (21.29%) and Leucocytozoon sp. (2.16%) were identified either in single or mixed infection. According to age, highest prevalence was recorded in adult (61.81%) and lowest in squab (36.25%). Comparatively, infection was recorded higher in females (58.22%) than males (48.79%). Season wise, infection was recorded highest during Pre-monsoon (72.22%) and lowest during Post-monsoon. Amplification of cyt b gene of Haemoproteus columbae in positive samples by PCR showed clear band at 207 bp. Amplification of mt- cyt b gene of Haemoproteus spp. and Plasmodium spp. by PCR on positive samples revealed clear band at 525 bp.

Keywords
Pigeon, Haemoprotozoa, Prevalence, PCR, Assam

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Introduction

Species of apicomplexan Haemoproteus, Plasmodium and Leucocytozoon are well known genera of avian haematozoa and comprise a diverse group of vector transmitted parasites.

They are closely related genetically but different in life history traits (Valkiunas, 1993). Avian malaria, caused by Plasmodium sp. is transmitted to birds by mosquitoes and has a long-term effect on the reproductive system of the host causing population decrease (Lapointe et al., 2012). Leucocytozoon sp. typically causes anaemia and enlargement of liver and spleen (Dey et al., 2010). Haemoproteus columbae commonly infect pigeon and doves and is widely distributed in tropical and subtropical regions and transmitted by blood sucking hippoboscid fly Pseudolynchia canariensis.

Its pathogenicity is generally low; however, due to acute infections in severely affected young pigeon heavy mortality is seen (Dey et al., 2010).
Materials and Methods

Study period

The present study was undertaken to ascertain the haemoprotozoan infection in domestic pigeon (Columba livia domestica) for a period of one calendar year w.e.f. February 2015 to January 2016.

Sample collection

Four districts of Assam namely Kamrup Rural, Kamrup Metro, Lakhimpur and Dhemaji formed the study areas. Blood samples of pigeons were collected from different households, market places and temple premises. The pigeons were categorized according to age viz. squab (< 30 days), young (30-90 days) and adult (> 90 days) and sex (male and female). The study period was divided into four seasons viz. Pre-monsoon (March, April, May), Monsoon (June, July, August, September), Post-monsoon (October, November) and Winter (December, January, February).

Sampling of Blood for Detection of Haemoprotozoa

Blood samples from 324 live pigeons were collected from wing vein using a 2 ml disposable syringe in EDTA vials and brought to the laboratory for parasitological and molecular analysis. For molecular analysis, the anticoagulated blood was stored in deep freeze at -20ºC until further use. The parasites were identified on the basis of their characteristic morphology (Levine, 1977; Soulsby, 1982) and percent parasitaemia (No. of parasitized cell /Total no. of respective cell x 100 = % parasitaemia) in positive cases were estimated.

Molecular detection of Haemoproteus columbae

DNA was extracted from 30 random positive samples of blood using DNeasy Blood and Tissue Kit (Qiagen Germany) as per manufacture’s guidelines. The extracted DNA was stored at -20º C until further use. The PCR was performed following the method of Doosti et al., (2014) to amplify a segment of cyt b gene of Haemoproteus columbae using oligonucleotide primer (H. clom- F 5′-TGA ACA TGC ATG CAA CTG GTG-3′ and H. clom-R 5′-TAG TAA TAA CAG TTG CAC CCC AG-3′) in 25µl reaction mixture containing 1µl DNA template, 1µl (20 pmol/µl) of each forward and reverse primer, 1µl MgCl2 (50 mM), 0.5µl dNTPs mix (10 mM), 0.25µl Taq DNA polymerase (5 IU/ µl) and the remaining volume adjusted with nuclease free water. PCR amplification was performed in a Technee-5000 thermal cycler (Bibby Scientific). PCR was performed with Initial denaturation at 94°C for 5 min followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 60º C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. A negative control consisting of a reaction mixture without the DNA was used.

Molecular detection of Haemoproteus spp. and Plasmodium spp.

DNA was extracted from 10 random positive blood samples of pigeons having simultaneous infection of Haemoproteus columbae and Plasmodium relictum on blood smear examination using DNeasy Blood and Tissue Kit (Qiagen Germany). PCR was
performed following the method described by Valkiunas et al., (2008) with little modification to amplify a segment of mitochondrial cyt b gene of Haemoproteus spp. and Plasmodium spp. using oligonucleotide primers (Haem F 5'-ATGGTGCTTTCGATATATGCATG-3' and (HaemR2 5'-GCATTATCTGGATGATAATGGT-3'). PCR amplification was done in a Technee-5000 thermal cycler (Bibby Scientific) in 25µl reaction mixture containing 5 µl of genomic DNA, 2.5 µl 10x PCR buffer, 1.0 µl MgCl₂ (50 mM), 0.5 µl dNTP (10 mM), 1.0 µl (20 pmol/ µl) of each forward and reverse primer, 0.2 µl Taq DNA polymerase (5 IU/ µl) and nuclease free water up to 25 µl. PCR amplification was done with initial denaturation at 94˚C for 3 min, and then 35 cycles consisting of denaturation for 30 sec at 94˚C, annealing for 30 sec at 50˚C and extension for 45sec at 72˚C, followed by final extension at 72˚C for 10 min. A negative control consisting of a reaction mixture without the DNA template was taken.

**Electrophoresis**

For visualization of the PCR product, gel electrophoresis of amplified DNA was done in 1.5 % agarose gel for 1 hour at 5 Volts per cm using 1 X Tris Acetate EDTA (1X TAE) running buffer. Four µl of the PCR product mixed with 3 µl of gel loading dye (6X DNA Loading Dye, Fermentas) was loaded on to the gel with standard markers (100 bp DNA ladder, Fermentas). The gel was then stained with ethidium bromide (0.5 µg/ ml) and visualized under gel doc (DNR Bio-Imaging System, Mini Lumi) for the expected product size and images were obtained.

**Statistical analysis**

Chi-square test was used for statistical analysis of the prevalence data using SAS v.20 software.

**Results and Discussion**

**Prevalence of haemoproteozoa according to parasite species**

Species wise, prevalence of Haemoproteus columbae was 29.93%, Plasmodium relictum 21.29% and Leucocytozoon sp. 2.16% (Table 1 and Fig. 1) without significant statistical difference (P<0.05).

Prevalence of H. columbae ranging from 15 to 80% was reported by several workers (18% by Ishtiaq et al., 2007 from India; 22% by Valkiunas et al., 2008; 47.05% by Radfar et al., 2011 from Iran; 60% by Roy et al., 2011 from Assam; 69.09% by Varshney et al., 2014 from Surat; 74.28% Borkataki et al., 2015 from Jammu). Report of 28% prevalence by Abed et al., (2014) from Iraq is in agreement with our findings. Studies to date have reported that the most common blood parasite found in pigeons is H. columbae, usually considered to be non-pathogenic but may cause disease in stressed pigeons. The variation in prevalence rate of this parasite in different countries might be influenced by geographical region, vector abundance, host genotype, host size, age or sex of host, feeding habitats, health status of bird etc.

Prevalence of Plasmodium relictum (27.5%) similar to our findings was reported from Kamakhya premises, Assam by Roy et al., (2011) and Gupta et al., (2011) from Uttar Pradesh (6.76%). This finding substantiates that mosquito of Genus Culex, the vector of pigeon malaria is commonly prevalent in Assam.

Prevalence of Leucocytozoon sp. in the present finding is similar to the report of 2% by Nath et al., (2014) from Bangladesh. However, higher prevalence has been reported by several workers (6.4% by Natala
et al., 2009; 20% by Dey et al., 2010 and 25% by Valkiunas et al., 2008) which contradict our findings and possibly it might be due to study made in different environments, population of vector fly and number of birds examined. In the present study, prevalence of mixed infection of Haemoproteus columbae and Plasmodium relictum was recorded as 7.71%. Beadell et al., (2009) similarly reported 6.8% pigeons in the Australo-Papuan region. Contrary to our finding, slightly lower prevalence (2.67%) was reported by Jahan et al., (2011) from Uttar Pradesh. Co-infection with two or more parasites revealed that the presence of one haemoparasite predisposes to other haemosporidian infections. Our finding agrees with the above statement. There was a noticeable relationship between the prevalence of H. columbae (29.93%) and its vector, Pseudolynchia canariensis (15.12%). The closeness in their percentage prevalence suggests that most of the vector harboured by the pigeons were probably carrying pathogens. According the Taylor et al., (2007), the presence of Plasmodium and Leucocytozoon in the blood of the pigeons was an indication of the presence of Culex and Simulium respectively, as they are established vectors of these haemoparasites. In our study of haemoproteozoa, the mean concentration of parasites was 1-6 pars/100 RBC for both H. columbae and P. relictum with variation in the shape and size of the gametocytes (Fig. 5). Similar reports were made (Gupta et al., 2011; Jahan et al., 2011 and Hussein et al., 2016) from India and elsewhere.

Age wise prevalence of haemoproteozoa parasites

The present finding recorded 61.81% prevalence of haemoproteozoa in adult followed by young (56.71%) and squab (36.25%) (Table 2 and Fig. 2) with statistical significance (P<0.05). Our report conform that of Momin et al., (2014) from Bangladesh who stated that adults were 6.89 times more susceptible than young birds. Msoffe et al., (2010) from Tanzania also made identical report. It is apprehended that adult birds are generally more attacked by vector flies.

Sex wise prevalence of haemoproteozoa parasites

Sex wise, prevalence was recorded more in female (58.22%) than the male (48.71%) (Table 3, Fig. 3) with non-significant (P>0.05) difference and agreeing with the findings of Momin et al., (2014). However, several workers from abroad (Dey et al., 2010; Opara et al., 2012 and Hussein et al., 2016) recorded higher prevalence in male than female. Though the exact cause of higher infection in females could not be explained it was assumed due to higher level of prolactin and progesterone suppressing the immune system of the individual and making the female more susceptible to any infection.

Seasonal prevalence of haemoproteozoa

Haemoproteozoa infection was recorded highest during Pre-monsoon season (72.22%) and lowest during Post monsoon, however, infection was more or less present throughout the year (Table 4 and Fig. 4). It might be due abundance of vector in Pre monsoon season. Literature is scant leading to less information on this aspect.

Molecular detection of Haemoproteus columbae

PCR employed for molecular detection of H. columbae by amplification of cyt b gene showed clear band at 207 bp (Fig. 6a) similar to the work of Doosti et al., (2014) who reported 23.18% prevalence of H. columbae in 220 pigeons in Iran.
Table 1: Species-wise prevalence of haemoproteozoa in pigeon

| Parasite species          | Sample examined (n=324) |  |  |  | Chi-square value |
|---------------------------|-------------------------|---|---|---|-------------------|
|                           | Single infection        | Mixed infection            | Total |
|                           | No. (%)                 | No. (%)                    | No. (%) |
|                           | H. columbae             | P. relictum                | Leucocytozoon sp. |
| *Haemoproteus columbae*   | 69 (21.29)              | -                          | 25 (7.71)   |
|                           |                         | 3 (0.92)                   | 97 (29.93)  |
| *Plasmodium relictum*     | 44 (13.58)              | 25 (7.71)                  | -           |
|                           |                         | 0 (0.0)                    | 69 (21.29)  |
| *Leucocytozoon sp.*       | 4 (1.23)                | 3 (0.92)                   | 0 (0.0)     |
|                           |                         | -                          | 7 (2.16)    |
| Overall                   | 117 (36.11)             | 28 (8.64)                  | 25 (7.71)   |
|                           |                         | 3 (0.92)                   | 173 (53.39) |

*P(<0.05)

Table 2: Age wise prevalence of haemoproteozoon parasites in pigeon

| Age group (No. examined) | Parasite Prevalence | Total |
|--------------------------|---------------------|-------|
|                          | *Haemoproteus columbae* |       |
|                          | No. positive (%)     |       |
| Squab (80) (<30 days)    | 15 (18.75)           | 29 (36.25) |
| Young (134) (30-90 days) | 46 (34.32)           | 76 (56.71) |
| Adult (110) (>90 days)   | 36 (32.72)           | 68 (61.81) |
| Total (324)              | 97 (29.93)           | 173 (53.39) |

*P(<0.05)

Table 3: Sex-wise prevalence of haemoproteozoon parasites in pigeon

| Sex          | No. examined | *Haemoproteus columbae* | Plasmodium relictum | Leucocytozoon sp. | Total |
|--------------|--------------|-------------------------|---------------------|-------------------|-------|
|              | No. Positive (%) | No. positive (%) | No. positive (%) | No. positive (%) |
| Male         | 166          | 42 (25.30)             | 36 (21.68)          | 3 (1.80)          | 81 (48.79) |
| Female       | 158          | 55 (34.81)             | 33 (20.88)          | 4 (2.53)          | 92 (58.22) |
| Total        | 324          | 97 (29.93)             | 69 (21.29)          | 7 (2.16)          | 173 (53.39) |

*NS (Non significant), P>0.05
Table 4 seasonal prevalence of haemoprotozoa in pigeon

| Month/season                  | Samples screened for haemoprotozoa | Sample positive for haemoprotozoa (%) | Chi-square value |
|-------------------------------|------------------------------------|--------------------------------------|------------------|
| Premonsoon (March, April, May) | 90                                 | 65 (72.22%)                          |                   |
| Monsoon (June, July, August, September) | 78                                | 36 (46.15%)                          |                   |
| Post monsoon (October, November) | 56                                 | 30 (53.57%)                          | 6.7118<sup>NS</sup> |
| Winter (December, January, February) | 100                                | 42 (42.0)                            |                   |
| Total                         | 324                                | 173 (53.39%)                         | (Non-significant) P(>0.05)

Fig. 1 Species-wise prevalence of haemoprotozoa in pigeons

- **Haemoproteus columbae**: 29.93%
- **Plasmodium relictum**: 21.29%
- **Leucocytozoon sp.**: 2.16%
Fig. 2 Age-wise prevalence of haemoprotozoa in pigeons

Fig. 3 Sex-wise prevalence of haemoprotozoan parasites in pigeon

Fig. 4 Seasonal prevalence of haemoprotozoa in pigeon
**Fig.5** Immature stages (gametocytes) (a-b), mature gametocytes (c-f), of *Haemoproteus columbae*; mature gametocytes (g-h), of *Plasmodium relictum* 1000X (Oil immersion)
**Fig.6** (a) PCR product at 207 bp of *Haemoproteus columbae* (L-Ladder 100 bp, Lane-1, 2, 3, 4, 5, 6 & 8- positive sample, 7- Negative control) & b) PCR product at 525 bp of *Haemoproteus* and *Plasmodium* (L-Ladder:100 bp, Lane-1, 2, 3, 4 & 5- positive samples and 6-Negative control)

**Molecular detection of *Haemoproteus* spp. and *Plasmodium* spp.**

PCR employed for simultaneous detection of *H. columbae* and *P. relictum* by amplification of mt-cyt b gene revealed clear band at 525 bp (Fig.6b). In our present study, by microscopic examination some early developmental stages of *Haemoproteus columbae* and *Plasmodium relictum* could not be morphologically differentiated, especially in mixed infection. However, it was confirmed by PCR. Similarly, Hellgren *et al.*, (2004) opined that by conventional microscopy, especially in chronic infections, species of *Haemoproteus* might be difficult to distinguish from avian
species of *Plasmodium*. Several PCR-based methods for studies of *Haemoproteus* spp. and *Plasmodium* spp. have been reported (Bensch et al., 2000; Richard et al., 2002; Bell et al., 2015). Similarly, Hellgren et al., (2004) and Bell et al., (2015) described a Nested PCR assay targeting the *cyt b* gene of the parasites, for screening and typing of *Leucocytozoon* sp. in parallel with *Haemoproteus* and *Plasmodium* in avian blood samples. From the present study, it was found that 53.39% pigeon were infected with three types of blood protozoa such as *Haemoproteus columbae* (29.93%), *Plasmodium relictum* (21.29%) and *Leucocytozoon* sp. (2.16%). It may be concluded that the protozoan infections in pigeon are highly endemic in Assam. The systematic study conducted for the first time in Assam led to a significant conclusion that favourable climatic condition and presence of vectors are the contributing factors towards prevalence of haemoprotozoan parasites.

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