Int.J.Curr.Microbiol.App.Sci (2019) *8*(5): 1426-1436

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 05 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.805.163

Prevalence and Molecular Detection of Blood Protozoa in Domestic Pigeon

Munmi Saikia[1]*, Kanta Bhattacharjee[1], Prabhat Chandra Sarmah[1], Dilip Kr. Deka[1], Shantanu Tamuly[2], Parikshit Kakati[1] and Pranab Konch[3]

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

**Corresponding author*

Keywords

Pigeon, Haemoprotozoa, Prevalence, PCR, Assam

Article Info

Accepted:
12 April 2019
Available Online:
10 May 2019

A B S T R A C T

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.

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 –20 ºC until further use thin blood smears were prepared using commercial Giemsa stain and examined under high power (40X) and oil immersion objective (100X) of light microscope for detection of *Haemoproteus* sp. and *Plasmodium* sp. inside the red blood cells and *Leucocytozoon* sp. inside the lymphocytes and monocytes. 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′-TTA GAT 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 $MgCl_2$ (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′-GCATTATCTGGATGTGATAATGGT-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 $MgCl_2$ (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 haemoprotozoa 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 haemoprotozoa, 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 haemoprotozoan parasites

The present finding recorded 61.81% prevalence of haemoprotozoa 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 haemoprotozoa 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 haemoprotozoa

Haemoprotozoan 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 haemoprotozoa in pigeon

Parasite species	Sample examined (n=324)					Chi-square value
	Single infection No. (%)	Mixed infection No. (%)			Total No. (%)	
		H. columbae	*P. relictum*	*Leucocytozoon* sp.		
Haemoproteus columbae	69 (21.29)	-	25 (7.71)	3 (0.92)	97 (29.93)	23.6716*
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 haemoprotozoan parasites in pigeon

Age group (No. examined)	Parasite Prevalence			Total No. (%)	Chi-square value
	Haemoproteus columbae No. positive (%)	*Plasmodium relictum* No. Positive (%)	*Leucocytozoon* sp. No. Positive(%)		
Squab (80) (< 30 days)	15 (18.75)	14 (17.50)	0 (0.0)	**29 (36.25)**	24.6516*
Young (134) (30-90 days)	46 (34.32)	26 (19.40)	4 (2.98)	**76 (56.71)**	
Adult (110) (>90 days)	36 (32.72)	29 (26.36)	3 (2.72)	**68 (61.81)**	
Total (324)	**97 (29.93)**	**69 (21.29)**	**7 (2.16)**	**173 (53.39)**	

* P(<0.05)

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

Sex	No. examined	***Haemoproteus columbae***	***Plasmodium relictum***	***Leucocytozoon* sp**	Total	Chi-square value
		No. Positive (%)	No. positive (%)	No. positive (%)	No. positive (%)	
Male	166	42 (25.30)	36 (21.68)	3 (1.80)	81 (48.79)	1.3215^{NS}
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%)	6.7118^{NS}
Monsoon (June, July, August, September)	78	36 (46.15)	
Post monsoon (October, November)	56	30 (53.57)	
Winter (December, January, February)	100	42 (42.0)	
Total	**324**	**173 (53.39)**	

^{NS}(Non-significant) P(>0.05)

Fig.1 Species-wise prevalence of haemoprotozoa in pigeons

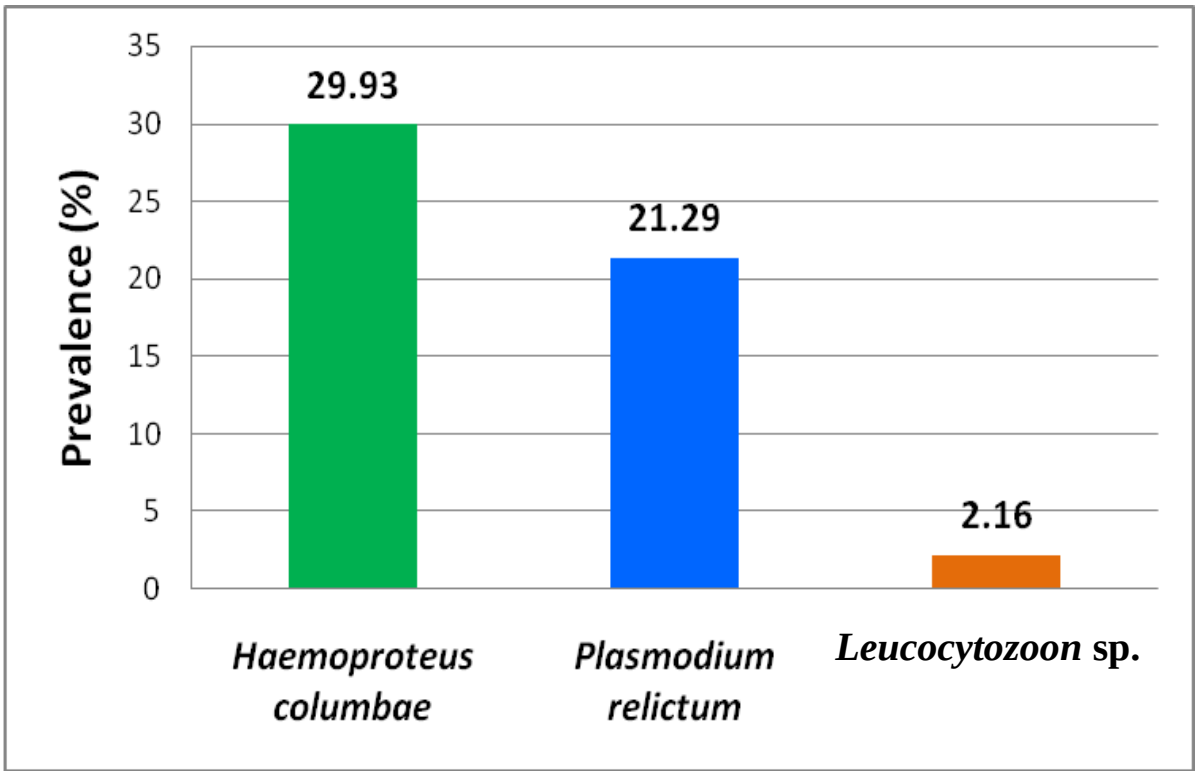


Fig.2 Age-wise prevalence of haemoprotozoa in pigeons

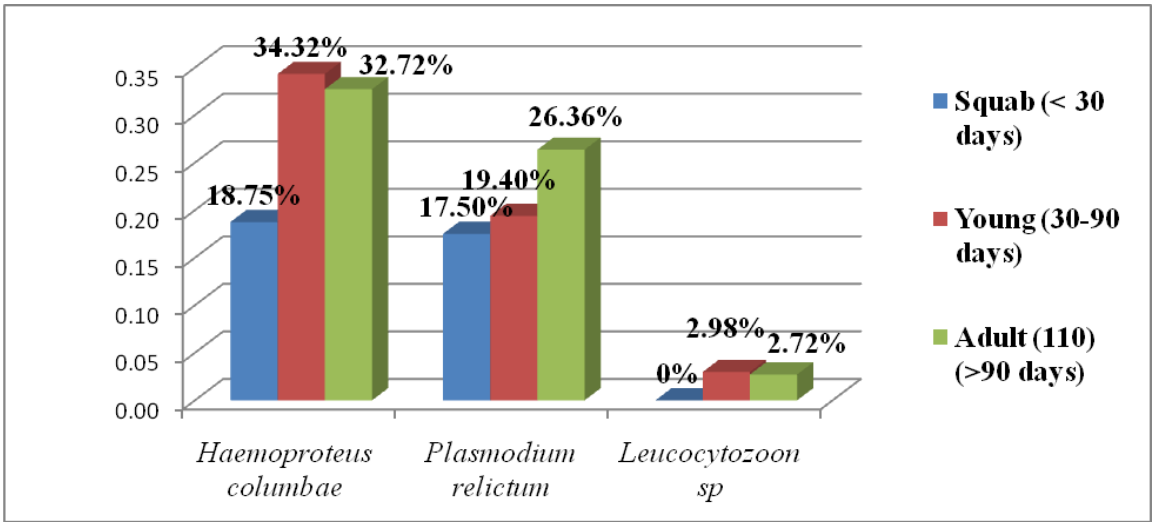


Fig.3 Sex-wise prevalence of haemoprotozoan parasites in pigeon

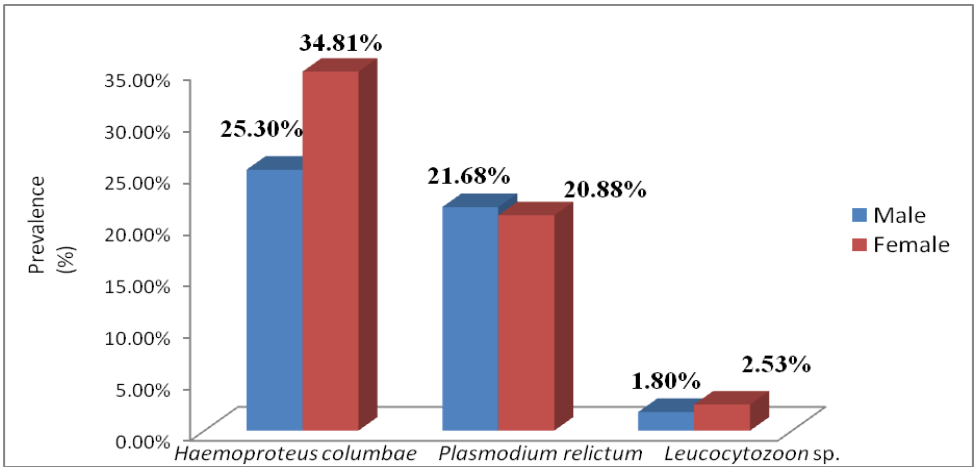


Fig.4 Seasonal prevalence of haemoprotozoa in pigeon

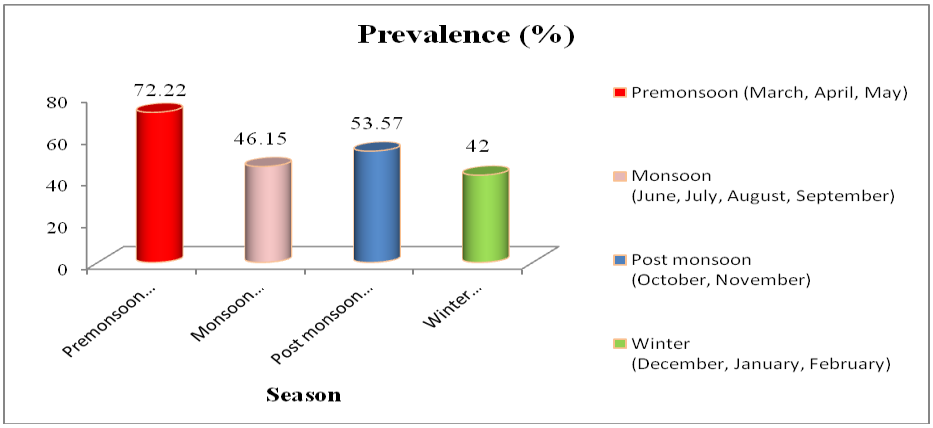


Fig.5 Immature stages (gametocytes) (a-b), mature gametocytes (c-f), of *Haemoproteus columbae*; mature gametocytes (g-h), of *Plasmodium relictum* 1000X (Oil immersion)

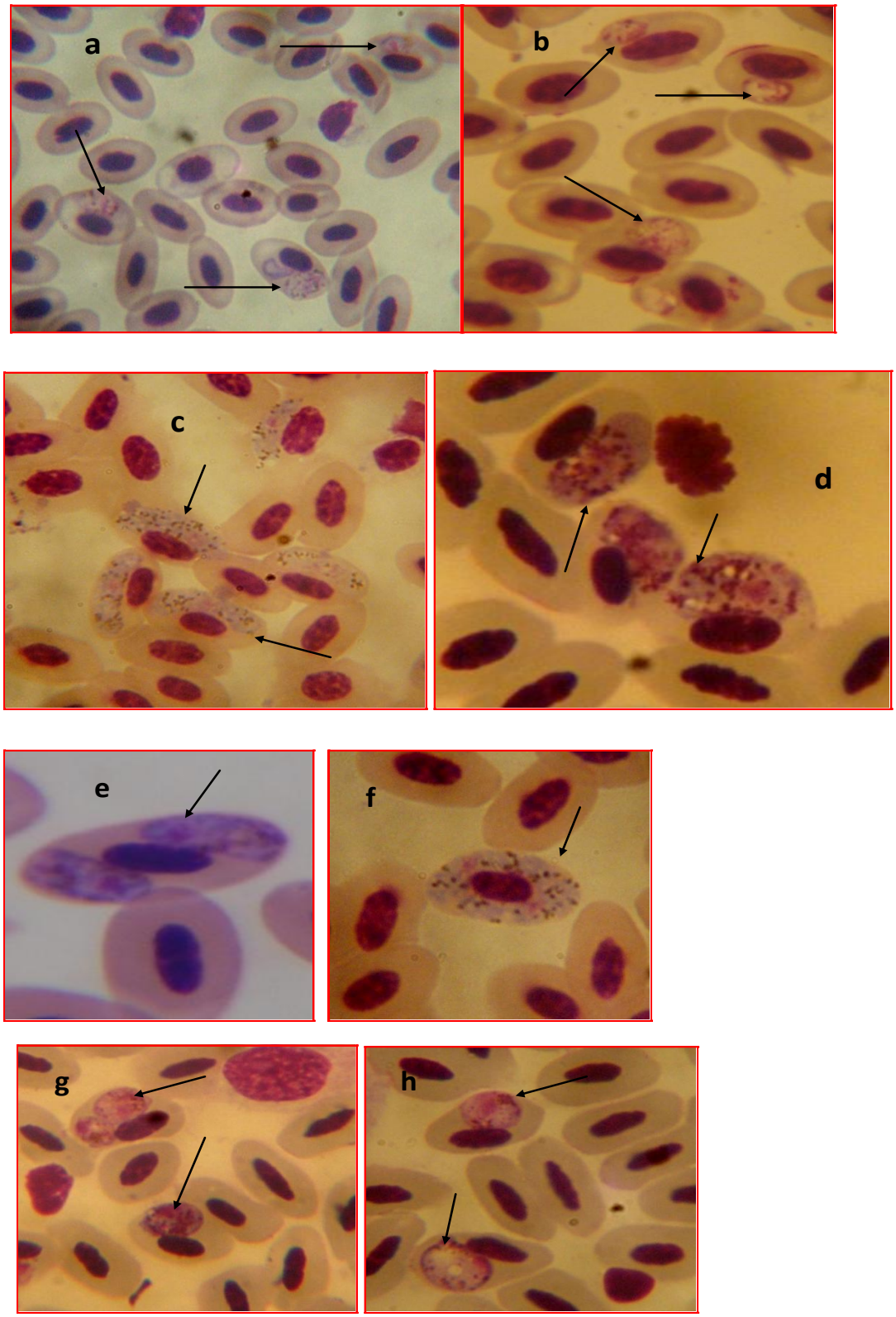
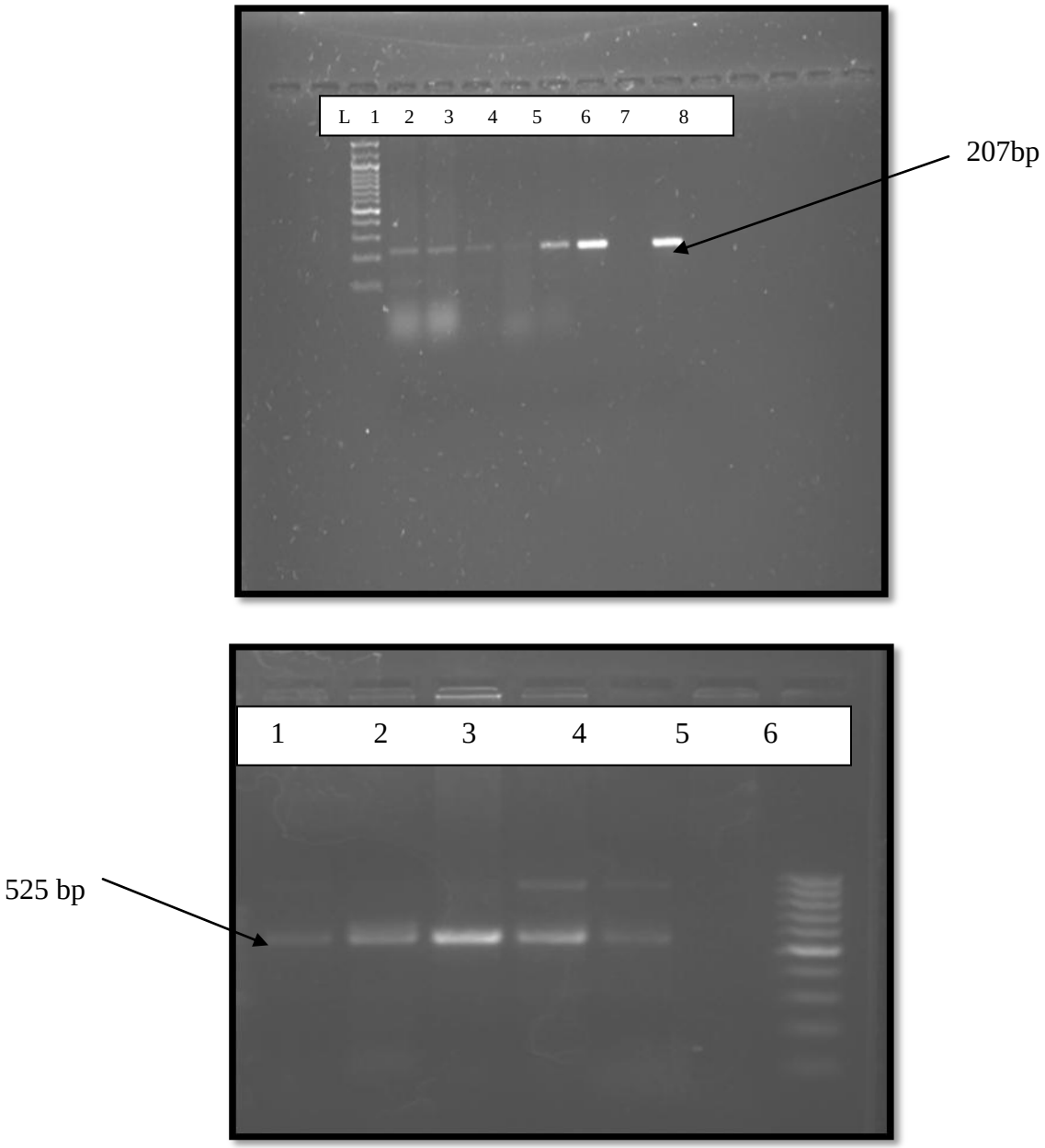


Fig.6 (a) PCR product at 207 bp of *Haemoproteus columbae* (L-Ladder 100 bp, Lane-1, 2, 3, 4, 5, 6 & 8- positive sample, 7- Negative control) & b) PCR product at 525 bp of *Haemoproteus* and *Plasmodium* (L-Ladder:100 bp, Lane-1, 2 , 3, 4 & 5 -positive samples and 6-Negative control)



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.

Acknowledgement

The authors are thankful to the Dean, College of Veterinary Science, Assam Agricultural University for providing the necessary facilities to conduct the study.

References

Beadell, J.S., Covas, R., Gebhard, C., Ishtiaq, F., Melo, M. and Schmidt, B.K. 2009. Host associations and evolutionary relationships of avian blood parasites from West Africa. *International Journal of Parasitology*. 39: 257-266.

Bell, J. A., Weckstein, J. D., Fecchio, A. and Tkach, V.V. 2015. A new real- time PCR protocol for detection of avian haemosporidians. *Parasites & Vectors.* 8:383.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H. and Pinheiro, R.T. 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings of the Royal Society of London.* 267:1583-1589.

Borkataki, S., Katoch, R., Goswami, P., Godara, R., Khajuria, J. K., Yadav, A., Kour, R. and Mir, I. 2015. Incidence of *Haemoproteus columbae* in pigeons of Jammu district. *Journal of Parasitic Diseases.* 39 (3): 426-428.

Dey, A.R., Begum, N., Paul, S.C., Noor, M. and Islam, K.M. 2010. Prevalence and pathology of blood protozoa in pigeons reared at Mymensingh district, Bangladesh. *International Journal of Biological Research*. 2 (12): 25-29.

Doosti, A., Ahmadi, R., Mohammadalipour, Z. and Zohoor, A. 2014. Detection of *Haemoproteus columbae* in Iranian pigeons using PCR. International Conference on Biological, Civil and Environmental Engineering. March 17-18, Dubai (UAE).

Gupta, D.K., Jahan, N. and Gupta, N. 2011. New records of *Haemoproteus* and *Plasmodium* (Sporozoa: Haemosporida) of rock pigeon (*Columba livia*) in India. *Journal of Parasitic Diseases*. 35(2):155–168.

Hellgren, O., Waldenstrom, J. and Bensch, S. 2004. A new PCR for simultaneous studies of *Leucocytozoon*, *Plasmodium* and *Haemoproteus* from avian blooid. *Journal of Parasitology*. 90 (4): 797-802.

Hussein, M.N. and Abdelrahim, A. E. 2016. *Haemoproteus columbae* and its histopathological effects on Pigeons in Quena Governorate, Egypt. *IOSR Journal Pharmacy and Biological Sciences* (*IOSR-JPBS*). 11(1):79-90.

Ishtiaq, F., Gering, E., Rappole, J. H., Rahmani, A. R., Jhala, Y.V., Dove, C.J., Milensky, C., Olson, S. L., Pierce, M.A. and Fleischer, R. C. 2007. Prevalence and diversity of avian haematozoan parasites in Asia: A regional survey. *Journal of Wildlife Diseases.* 43(3): 382-398.

Jahan, N., Chandra, R. and Shoeb, M. 2011. Parasitimic load of haematozoan parasites

in Rock pigeons (*Columba livia*). *Recent Research in Science and Technology*. 3(6): 9-11.

Lapointe, D.A., Atkinson, C.T. and Samuel, M.D. 2012. Ecology and conservation biology of avian malaria. *Annals of the New York Academy of Sciences*. 1249: 211-226.

Levine, N.D. 1977. Textbook of Veterinary Parasitology. Burgess Publishing Company, USA.

Momin, M. A., Begum, N., Dey, A. R., Paran, M. S. and Alam, M. Z. 2014. Prevalence of blood protozoa in poultry in Tangail, Bangladesh. *IOSR Journal of Agriculture and Veterinary Science.* 7(7): 55-60.

Msoffe, P. L. M., Muhairwa, A. P., Chiwanga, G. H. and Kassuku, A. A. 2010. A study of ecto- and endoparasites of domestic pigeons in Morogoro Municipality, Tanzania. *African Journal of Agricultural Research.* 5 (3):264-267.

Natala, A.J., Asemadahun, N.D., Okubanjo, O.O. and Ulayi, B.M. 2009. A survey of parasites of domesticated pigeon (*Columba livia domestica*) in Zaria, Nigeria. *International Journal of Soft Computing*. 4 (4): 148-150.

Nath, T. C., Bhuiyan, M. J. U. and Alam, M. S. 2014. A study on the presence of leucocytozoonosis in pigeon and chicken of hilly districts of Bangladesh. *Issues in Biological Sciences and Pharmaceutical Research.* 2 (2):013-018.

Opara, M. N., Ogbuewu, I. P., Njoku, L., Ihesie, E.K. and Etuk, I.F. 2012. Study of the haematological and biochemical values and gastrointestinal and haemoparasites in racing pigeons (*Columba livia*) in Owerri, Imo State, Nigeria. *Revista Cientifica UDO Agricola.*12(4):955-959.

Radfar, M.H., S, Fathi., Asl, E. N., Dehaghi, M.M. and Seghinsara, H.R. 2011. A survey of parasites of domestic pigeons (*Columba livia domestica*) in South Khorasan. *Iranian Journal of Veterinary Research.* 4(1):18-23.

Richard, F.A., Sehgal, R. N. M., Jones, H. I. and Smith, T.B. 2002. A Comparative Analysis of PCR-Based Detection Methods for Avian Malaria. *Journal of Parasitology*. 88 (4):819-822.

Roy, K., Bhattacharjee, K. and Sarmah, P. C. 2011. Prevalence of endoparasites in pigeons. *Journal of Veterinary Parasitology*. 25: 94-95.

Soulsby, E.J.L. Helminths, Arthropods and Protozoa of Domesticated Animals. Seventh Edition, The English Language Book Society and Baillier Tindal and Cassel Ltd., London. 2012; 367-370, 440-441.

Taylor, M.A., Coop, R.L. and Wall, R. L. 2007.Veterinary Parasitology. 3rd Edn. Publ. Blackwell Ltd. Oxford. 1-874.

Valkiunas, G. 1993. Pathogenic influence of haemosporidians and trypanosomes on wild Birds in the field conditions. Facts and hypothesis. *Ecologija.* 1:47-60.

Valkiunas, G., Lezhova, A.T., Krizanauskiene, A., Palinauscas, V., Sehgal, N.M. R. and Bensch, S. 2008. A comparative analysis of microscopy and PCR- based detection methods for blood parasites. *Journal of Parasitology.* 94(6): 1395-1401.

Varshney, J. P., Deshmukh, V.V. and Chaudhury, P. S. 2014. Pseudomalaria (*Haemoproteus columbae)* in Pigeon Shelter. *Intas Polivet.* 15 (1):176-177.

How to cite this article:

Munmi Saikia, Kanta Bhattacharjee, Prabhat Chandra Sarmah, Dilip Kr. Deka, Shantanu Tamuly, Parikshit Kakati and Pranab Konch. 2019. Prevalence and Molecular Detection of Blood Protozoa in Domestic Pigeon. *Int.J.Curr.Microbiol.App.Sci.* 8(05): 1426-1436.
doi: https://doi.org/10.20546/ijcmas.2019.805.163