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Conventional and Molecular Detection of *Plasmodium* in Domestic Poultry Birds

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Abstract: The present study was conducted to detect the *Plasmodium*, the causative agent of malaria in domestic poultry birds. Blood smear method was used as the conventional method for the detection, whereas the polymerase chain reaction (PCR) was performed for further confirmation. A total of 50 blood samples were collected from poultry birds showing the malarial symptoms. The results of blood smear methods showed two samples (4%) were infected with genus *Plasmodium*, whereas the PCR analysis showed four (8%) positive samples. These results confirm that the PCR is more sensitive method for detecting the *Plasmodium* when compared with conventional methods, and the microscopy diagnosed 50% false negative results that were confirmed by PCR.

Key words: Poultry, *Plasmodium*, polymerase chain reaction, microscopy.

1. Introduction

Malaria, sometimes also called “king of diseases”, is a serious infection of blood caused by a single celled notorious parasite, i.e., *Plasmodium* that is known to affect different vertebrate hosts. Avian malaria parasites of the genus *Plasmodium* (Haemosporida: Plasmodiidae) are widespread on all continents, except Antarctica. Species of avian malaria parasites (*Plasmodium*) are common, but their virulence has been insufficiently investigated in wild and domestic birds [1-3].

Parasitologists have founded approximately 15 genera within the order Haemosporidia (Phylum Apicomplexa) to contain more than 500 described species that infect squamate reptiles, turtles, birds and mammals, and use at least seven families of dipteran vectors [4].

Taxonomists have described more than 200 species of avian haemosporidians existing hundreds of bird species. Haemosporida are transmitted from the infected to uninfected birds by a variety of biting flies that serve as vectors, including mosquitoes, black flies, ceratopogonidae flies (biting midges or sand flies) and louse. Prognosis is not good and birds often die within hours of displaying symptoms. Avian malaria produces a wide range of effects in avian hosts, from no apparent clinical signs to severe anemia and death. *P. gallinaceum*, *P. juxtanucleare* and *P. durae* appear to be the most dangerous for poultry causing up to 90% mortality. This can be positively identified with blood smear.

Microscopy is an established, relatively simple technique that is familiar to most laboratories. In many developing countries, microscopy is not reliable, because the microscopists are insufficiently trained and supervised and are overworked. Besides, the microscopes and reagents are of poor quality, and often the supply of electricity is unreliable. Conversely, in non-endemic countries, laboratory technicians are often unfamiliar with malaria and may miss the parasites [5].

Tests based on polymerase chain reaction (PCR) for species-specific *Plasmodium* genome are more sensitive and specific than other tests, and it can be capable of detecting as few as 10 parasites/μL blood [6].

PCR is more sensitive and more reliable as

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compared to blood smear test, because PCR rules out the false positive or false negative results. Therefore, PCR is proven to be sensitive and easy method to confirm the infection and the clinical monitoring of treatment of malaria [7].

The present study was therefore designed to compare the reliability and sensitivity of blood smear method and PCR for detection of *Plasmodium*.

2. Materials and Methods

2.1 Sampling

Sampling area was located in Mirpur Khas district, Pakistan (Fig. 1). The blood was collected from domestic chicken *Gallus gallus domesticus*, which was known to be susceptible for *Plasmodium* infection. Total 50 blood samples were collected, each sample contained 0.5 mL blood and blood was collected from wing vein of the *Gallus gallus domesticus* birds in Eppendorf tube containing 50 μL of ethylenediaminetetraacetic acid (EDTA). Thin blood smears were prepared and fixed in absolute (100%) methyl alcohol on spot, to avoid destruction or shrinkage of erythrocytes. The blood film was stained with Giemsa as described by Iqbal et al. [8]. The whole blood samples were stored at -20 °C until extraction of DNA.

2.2 Making Blood Smears

The blood smears were made as described by Iqbal et al. [8]. Briefly, the slides were held by the edges to avoid the finger prints contamination on slide area where the blood smear was made. A drop of blood was placed toward one end of the microscopic slide and with the edge of spreader slide; the drop of blood was spread across the touching edge of the spreader slide at 45° angle. The spreader slide was quickly moved on the surface of microscopic slide. The smear on the slide was air dried.

Fig. 1 The sampling area of Mirpur Khas district, Pakistan.

2.3 Fixation of Blood Smears

After drying the blood smears and prior to applying a stain slide, they were fixed in 100% methyl alcohol for 1-2 min to prevent the water dissolving the hemoglobin out of the red blood cell (RBC) and elude the shrinkage or rupturing of RBC.

2.4 Staining

Giemsa stain was used for staining the blood smears. The staining of the slides was done in 10% solution of Giemsa stain for 5 min and the slides were rinsed with distilled water.

2.5 Examination of Blood Smears

Slides were examined at 40× and 100× objectives using oil immersion in a binocular electric microscope. The identification of parasite was done with the help of taxonomic keys. Microscopic (40× and 100× magnifications) photographs were taken for better understanding and reference.

2.6 Molecular Technique

DNA from blood samples was extracted using commercial kit (Gene-JET Genomic DNA Purification Kit #K0721, Thermo Scientific, USA) as per manufacturer's instructions.

2.7 DNA Quantification through Nano-Spectrophotometry

In order to quantify the exact quantity of nucleic acid for a particular reaction, the quantification of extracted genomic DNA was performed by Nano-drop spectrophotometer (Nano-drop 1000 spectrophotometer, Thermo Scientific, USA). An aliquot of 2 μL of nuclease-free water was added to wash the scale. To follow the step further, 2 μL of elution buffer was added to scale for blanking the results, and finally 2 μL of extracted genomic DNA was added to measure the quantity.

2.8 PCR Amplification

Infection of poultry with *Plasmodium* was detected by using PCR. The PCR was performed on the extracted DNA by using primers specific for the genes of *Plasmodium* as described by Aslan et al. [9]. Primer of *plasmodium* follows through 5'-to-3' sequence and gene location: forward primer 5'-TTAAAATTGTTGCAGTTAAAACG-3' (10 pM/μL), and reverse primer 5'-CCAGACAAATCATATTCACG-3' (10 pmol/μL). The PCR assay was done in a 25 μL reaction volume containing 12 μL GoTaq Green Master Mix (2× Promega) pH 8.3, which contain 400 μM deoxy adenine, 400 μM deoxy guanine, 400 μM deoxy cytocine, 400 μM deoxy thymine and 3 mM $MgCl_2$, 2 μL of each primer, 5 μL nuclease free water and 4 μL of DNA extracted from *Plasmodium*-infected erythrocytes. PCR reaction was performed in Applied Biosystem 2720 Thermal Cycler, and the cycling condition was as an initial hot start for 4 min at 94 °C, followed by 40 cycles with denaturation for 1 min at 94 °C, annealing for 1 min at 52 °C and extension for 1 min at 72 °C and a final extension for 7 min at 72 °C. Amplification product was analyzed by gel electrophoresis, using 2% Agrose containing 3 μL of ethidium bromide from 10 mg/mL stock solution and visualized by UV trans-illumination device.

2.9 Primer

Primers were purchased from Gene Link, USA through Worldwide Scientific Lahore, which were supplied in desalted and lyophilized form. The primers were reconstituted in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.5).

2.10 Preparation of Stock Solution of 100 pmol/μL (100 μM)

Gene Link provides the exact amount of each primer supplied on the tube. The amount of n-mole of

forward primers was 38.7 and reverse primer was 45.2, multiplied with 10 to get the volume of TE added in each tube and vortex properly for mixing and diluted 10 fold to prepare a 10 pmol/μL (10 μM).

3. Results

Malaria in birds has many similarities to malaria in humans. Insect vectors feed on the exposed flesh around the eyes, beak and legs, immediately after they infect a bird. Sporozoites invade the tissues (mesodermal) and reproduce for one or more generations, before they become merozoites. Merozoites enter the RBC and become mature infectious gametocytes. Birds show symptoms within 7 d, suffer from loss of appetite, fever, weakness, depression and shortness of breath. Blood circulation to organs may be impaired, and the liver and spleen may be enlarged, with anemia due to destruction of RBC. Birds are listless with puffy, almond-shaped eyes, may display difficulty with body balance or eye-sight, sometimes vomiting, and typically have a high temperature. The infected birds defecate with dull jade green color, which may be a potential symptom of malarial infection. In heavy infections, death is common [10]. The presence of *Plasmodium* was confirmed through multiplying *Plasmodium* inside erythrocytes. During the present study, the blood samples for microscopy revealed presence of meronts inside erythrocytes (Fig. 2).

The most commonly encountered haemo-parasite in domestic poultry was *Plasmodium*, which was detected in 8.0% of the blood samples. Two different techniques were applied to detect malaria. One was conventional microscopy, which detected malaria in domestic poultry birds. Total 50 blood samples collected from Mirpur Khas were subjected to microscopic examination, and out of 50 samples only two numbers of samples (4%) were found malaria positive (Table 1). Another technique was PCR. Same samples were subjected to PCR, and out of 50 samples four numbers of samples (8%) were found positive, having *Plasmodium* genomic DNA demonstrated in genomic bands at 420 bps (Table 2). When compiled both techniques, out of 50 processed samples of domestic poultry birds, two samples (4%) were found positive through microscopy and four samples (8%)

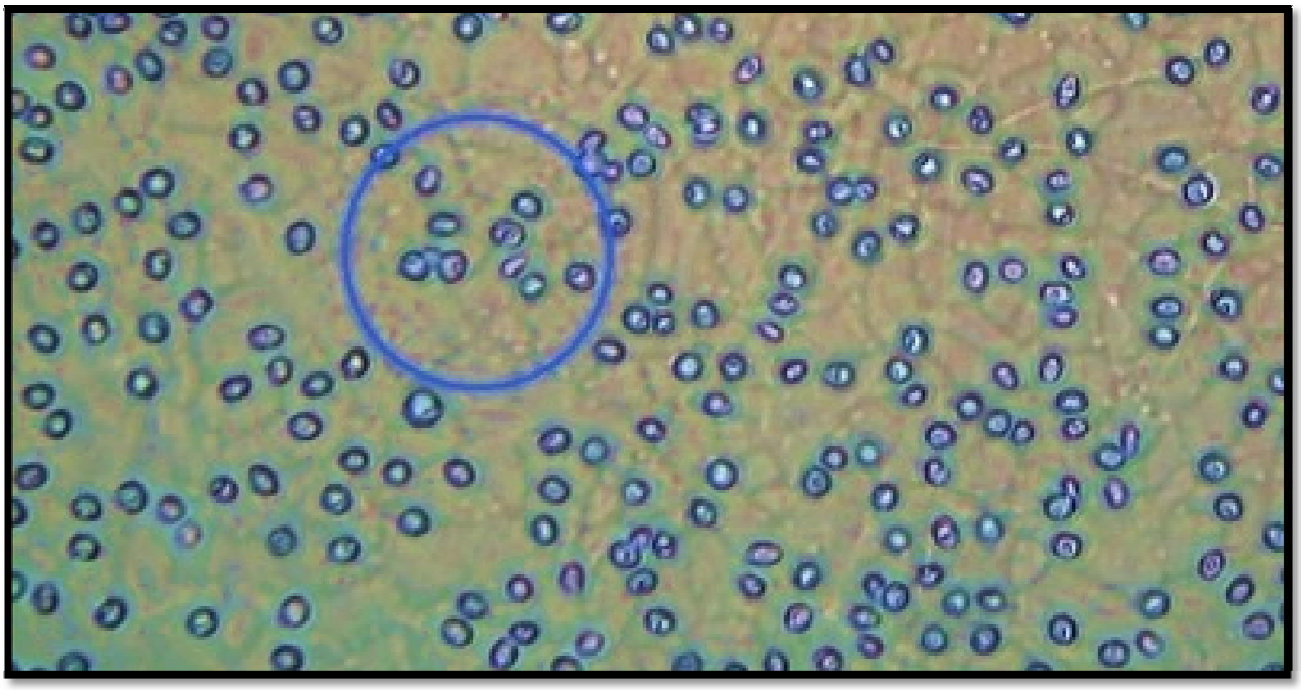

Fig. 2 The thin blood film with erythrocytic meronts.

Table 1 Prevalence percentage of malaria in domestic poultry birds examined through microscopy.

Location	No. of samples examined	No. of positive sample through microscopy	Percentage
Mirpur Khas	50	2	4%

Table 2 Prevalence percentage of malaria in domestic poultry birds examined through PCR.

Location	No. of samples examined	No. of positive sample through PCR	Percentage
Mirpur Khas	50	4	8%

Table 3 Comparison between microscopy and PCR results in domestic poultry.

Location	No. of samples examined	Microscopy positive	PCR positive	Comparison between microscopy and PCR
Mirpur Khas	50	2	4	2 (50%)

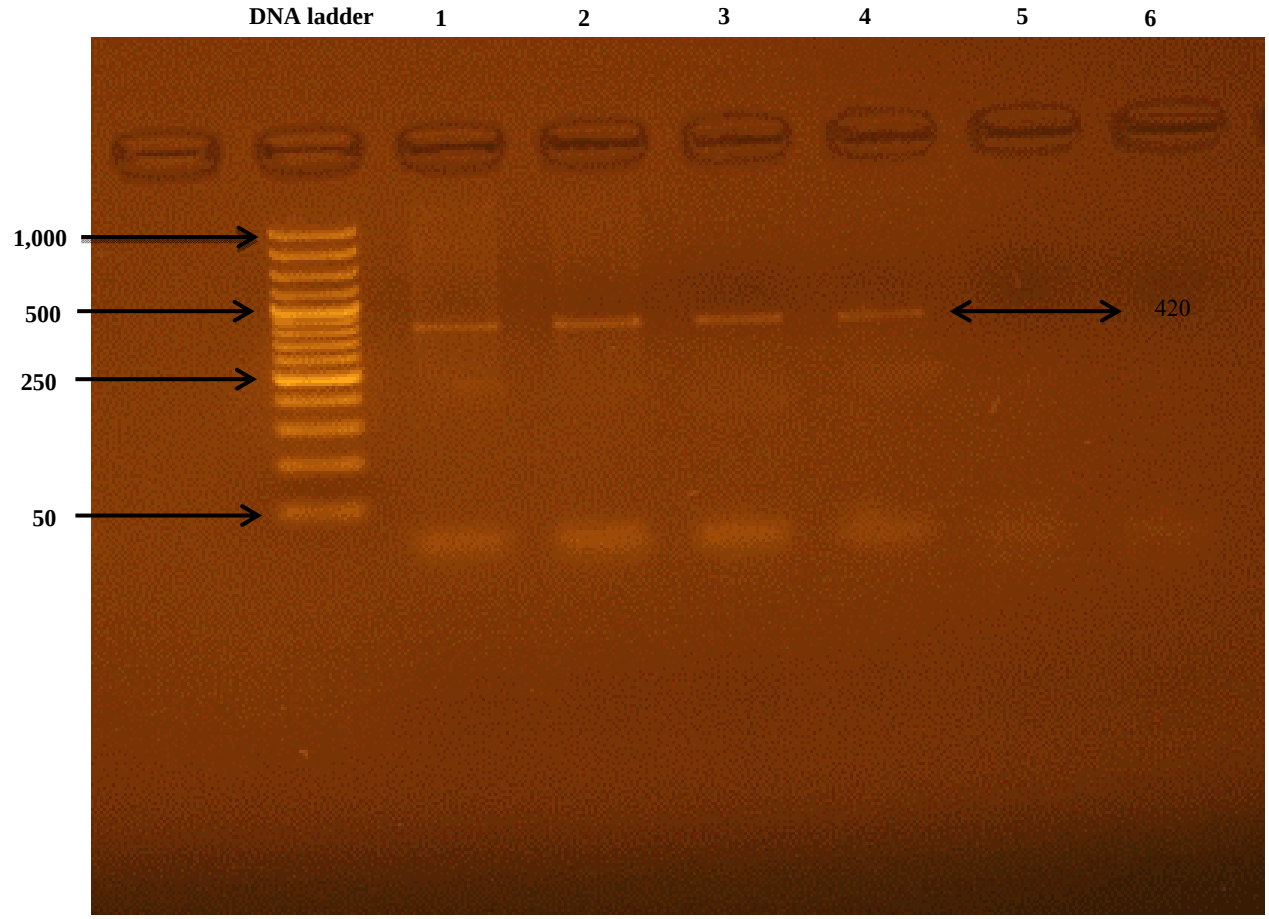

Fig. 3 PCR amplification product obtained from blood samples of domestic poultry birds having malarial symptoms.
DNA ladder of 100 bps; 1-4: positive for *Plasmodium* DNA; 5 and 6: negative for *Plasmodium* DNA PCR product, indicating DNA bands at 420 bps.

positive through PCR. Thus, there was 50% false negative results detected through microscopy, which was confirmed by PCR protocol (Table 3).

During the present study, domestic poultry birds samples were treated by routine microscopy and same samples were subjected to amplification of genomic DNA by PCR. The PCR results presented in Fig. 3 show the presence of genomic DNA bands at 420 bps. The PCR results confirm the prevalence of malaria in birds in Mirpur Khas district.

4. Discussion

Malaria, caused by unicellular protozoan of genus *Plasmodium*, is a disease transmitted by female mosquito and other biting flies. Avian malaria has a worldwide distribution and is endemic to parts of Asia including Pakistan. It is of great economic significance to the poultry industry. Organisms, such as *P. gallinaceum*, *P. juxtanucleare* and *P. durae*, may cause up to 90% mortality in poultry [11].

Microscopic detection of parasites on Giemsa-stained blood smears has been the reference standard method for malaria diagnosis laboratories for more than one century. It is a deficient standard, highly dependent on the technical expertise of the microscopists. Microscopic diagnosis sometimes may be misleading in identifying *Plasmodium* species, especially in cases with low level of parasitemia, a mixed parasitic infection, or when modified by antimalarial drug treatment [12]. Molecular or nucleic acid-based diagnostic methods for parasitic infections have been developed over the past 12 years, and the PCR assays proved to be valuable for epidemiological studies of parasites [13].

In the present study, 4% domestic poultry birds were infected with malaria through microscopy, while 8% through PCR. When compared both techniques, 50% results were detected false negative through microscopy, confirmed by PCR. Snounou et al. [14] reported that PCR was highly sensitive and accurate than routine diagnostic microscopy in detection and identification of parasites.

Prevalence of malaria in domestic poultry has not been studied in the present studied area. Saiwichai et al. [15] detected avian malaria, caused by *P. gallinaceum* in infected fresh-blood samples by nested PCR. Aslan et al. [9] compared PCR product with microscopy in malaria suspected cases in Turkey. They processed 114 positive blood samples, prepared thin and thick blood smears and also subjected samples to extraction of DNA. The obtained fragments were analyzed by agarose gel electrophoresis. The number of parasites in the thick and thin smears of the blood samples was evaluated microscopically after staining by Giemsa, and results were compared by PCR results. Among all 114 *Plasmodium* positive cases detected by microscopy, 100 were detected by PCR, revealing that 14 samples were false negative. In the present study, false negative were also observed in domestic poultry by microscopy.

It is concluded that conventional methods of diagnosis are not reliable, hence alternate options are tried to reach accurate conclusion. PCR has emerged as reliable technology in the world of diagnostics, as it rules out artefacts to be diagnosed as positive/negative. The developments of new PCR-based methods in the last years have an important advance in the study of infectious diseases. PCR assay was most sensitive and specific method to detect malaria parasites. However, the time lag between sample collection, transportation and processing, and dissemination of results back to the physician, limit the usefulness of PCR in routine clinical practice [16].

5. Conclusions

In the present study, results revealed that out of 50 samples collected from domestic poultry birds, two (4%) samples were found positive by microscopy, while same number of samples subjected to PCR, four (8%) were found positive. Comparison of two different techniques, i.e., conventional blood smear test and PCR, it is revealed that as compared to blood smear test, PCR is more sensitive, specific and reliable. On the basis of present findings, it is concluded that conventional methods of diagnosis are not reliable, hence alternate options are applied to reach the accurate conclusion.

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