



CONVENTIONAL AND MOLECULAR DETECTION OF PLASMODIUM IN HUMANS

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ABSTRACT

Conventional and molecular detection of plasmodium, causative agent of malaria was carried out, during 2014-15 aiming at comparing the sensitivity of both the protocols. For conventional diagnosis, thin blood smear from 50 malaria suspected victims from Umerkot, Tandojam and adjoining areas were prepared and stained with Romanovisky stain, whereas molecular confirmation on same sample was done by Polymerase Chain Reaction (PCR). For identification of species, restriction enzyme (AluI) analysis of the amplified product was performed. Blood smear examination indicated that 18 (36%) cases were positive with plasmodium parasite, whereas PCR analysis detected plasmodium at 850 base pairs (bps) showing that only 6 (12%) blood samples were infected with plasmodium. When PCR positive samples were subjected to AluI enzyme, the protocols revealed that all victims were positive with *Plasmodium falciparum*. Comparison of conventional and molecular techniques revealed that 12 (66.60%) false Positive cases were detected by microscopy, whereas PCR assay did not conform to these results. Statistical analysis on the prevalence of malaria revealed non-significant difference from Umerkot and Tandojam at $P > 0.05$ ($X^2 = 1.815$; $P = 0.1779$) with 1 degree of freedom (df) whereas PCR results demonstrated significant difference at $P < 0.05$.

Keywords: malaria, plasmodium, polymerase chain reaction

INTRODUCTION

Malaria, a mosquito-borne disease is a life-threatening infection of Red Blood Cells caused by intra-erythrocytic parasites that belong to genus plasmodium. The pathogen may affect variety of vertebrates including man. Malaria is considered a leading cause of death in humans. About 300-500 million people are reported to suffer from malaria each year, resulting in 1.5-2.7 million deaths annually (Muentener *et al.*, 1999). However, the number of malaria cases fell from an estimated 262 million globally in 2000 (range: 205-316 million), to 214 million in 2015 (range: 149-303 million), a decline of 18%. Most cases and deaths in 2015 are estimated to have occurred in the W.H.O. African Region (88%), followed by the W.H.O. South-East Asia Region (WHO, 2015). The estimated number of annual malaria cases in Pakistan is 1.5 million (W.H.O.,

2005). There are five major species of *Plasmodium*, transmitted by female anopheles mosquito, which is specific to human malaria. Malaria is not uncommon in Pakistan. There are reports of low percentage of malaria in Punjab and the Azad Jammu and Kashmir (AJK), moderate malarial infection in Sindh and Khyber-Pakhtunkhwa and highest cases of malarial infections have been reported from Baluchistan and FATA (World Malaria Report, 2009). Proper diagnosis has supreme importance towards malaria management and control. The global impact of malaria has developed interest in all around the world to develop a gold standard diagnostic methodology (Bell *et al.*, 2005). Malaria can be diagnosed by thin blood smear microscopy. Besides, urine and saliva of the malarial victim can also be checked for this purpose. Level of parasitemia may be measured by examination of at least 200 parasites/ μ l in a thin blood smear (TBS), occasionally these tests are negative in patients with high parasitemia, and their sensitivity below 100 parasites/ μ l is low (Hanscheid and Grobusch, 2002). Yet, testing malaria by the blood microscopy is not a dependable procedure, because blood smear diagnosis has many shortcomings as false positive cases are confused with malaria (Parn, 2009).

Diagnostic tests based on polymerase chain reaction (PCR) is highly sensible and specific than conventional blood smear tests and has been used for *P. vivax* (Torres *et al.*, 2006) and *P. falciparum* (Zalis *et al.*, 1996). It can detect as few as 10 parasites/ μ l blood (Warrell *et al.*, 1982). The PCR technique may also help to distinguish between various plasmodium species on the basis of diversity in DNA which are detected through Restriction Fragment Length Polymorphism (Jill *et al.*, 1999). In Pakistan, there are very few published reports (Ghanchi *et al.*, 2011) on detection of malaria using PCR. This may be because most laboratories do not have capacity for this protocol. Besides, Restriction Fragment Length Polymorphism (RFLP) technique is seldom employed for detecting molecular diversity of plasmodium in Pakistan therefore, small scale work was conducted to compare the reliability and sensitivity of Blood Smear Method and Polymerase Chain Reaction for detection of plasmodium and to identify plasmodium species using RFLP.

MATERIALS AND METHODS

Sampling

Sampling for present study was done from two areas viz. Tandojam, Umerkot and adjoining areas, Sindh Pakistan. A total of 50 blood samples were obtained and analyzed. Samples were gathered from patients suspected of having malaria. Three milliliters (ml) venous blood was collected in Ethylene Diamine Tetracetic Acid (EDTA) coated tubes. Thin blood smears were prepared and fixed in absolute (100%) alcohol on spot, to avoid destruction or shrinkage of erythrocyte(s). The blood film was stained with Giemsa as described by Iqbal *et al.* (2006). The whole-blood samples were stored at -20°C until extraction of DNA.

Examination of blood smears

Slides were examined at X40 and X100 objectives using oil immersion in a binocular electric microscope. The identification of parasite was done with the

help of taxonomic keys. Microscopic photographs at 100 x were also taken for better understanding and reference.

DNA extraction

DNA from blood samples was extracted using commercial kit (Gene-JET™ Genomic DNA Purification Kit #K0721, Thermo Scientific, USA) as per manufacturer's instructions, and quantified through Nano-Spectrophotometer (Thermo-scientific USA). Briefly, 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.

PCR amplification

Infection of humans with plasmodium was detected by using PCR which was performed on the extracted DNA by using primers specific for the genes of plasmodium as described by Gonul *et al.* (2007). Follows (5'-to-3' sequence and gene location): forward primer, (10 pM/µl) (5' TTAAAATTGTTGCAGTTAAAACG 3') and reverse primer (10 pM/µl) (5' CCAGACAAATCATATTCACG 3'). Primers were purchased from Gene link USA in desalted and lyophilized form and were reconstituted in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The PCR assay was done in a 25 µl reaction volume containing GoTaq Green Master Mix, (2X (Promega) pH 8.3 which contain (400 µM Deoxy Adenine, 400 µM Deoxy Guanine, 400µM Deoxy Cytocine, 400 µM Deoxy Thymine and 3 mM MgCL₂) 12 µl, 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 cycling condition were as an initial hot start for 4 minutes at 94°C, followed by 40 cycles with denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C and extension for 1 minute at 72°C and a final extension for 7 minutes 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.

Agrose gel preparation

Agrose gel (2%) was prepared by taking 10 ml TAE buffer (Rotiphorese^R Roth) dissolved in 490 ml of water. 50 ml from that solution was taken in conical flask and 1 g of Agrose was added into the sample which was boiled in microwave for 50 seconds. Three µl (10mg/ml) of Ethidium Bromide were added and left for some time to let the gel slightly cool. The gel was placed in gel casting tray with comb inserted in it and was left till the gel was completely set. TAE buffer (450) was added in electrophoresis tray and the gel casting tray was shifted into electrophoresis tray and the comb was removed.

Sample loading and gel documentation

Prior to loading of samples, 6 µl of DNA ladder was added into first trough, and 6 µl PCR product was loaded into remaining holes. The electrophoresis unit was connected with power supply set 75 volts for at least 30 minutes. Gel casting was removed from electrophoresis tray and gently placed into Gel Documentation

system (Clever Scientific, UK). The bands were photographed for record, reference and identification.

RFLP analysis

In RFLP analysis, the DNA sample was digested by restriction enzymes (AluI) obtained from Thermo Scientific, USA and the resulting restriction fragments were separated according to their lengths by gel electrophoresis as per manufacturer's instruction.

RESULTS

With view to generate baseline information on Plasmodium related parameters in humans, this study was conducted. Blood samples were collected and subjected to blood smear microscopy and PCR protocol for pathogen detection. Furthermore, restriction enzymes were also applied to identify specific species of genus Plasmodium infecting humans. The presence of Plasmodium was confirmed through presence of schizonts inside RBC(s). During present study, the blood samples for microscopy revealed presence of schizonts inside erythrocytes (Plate 1).

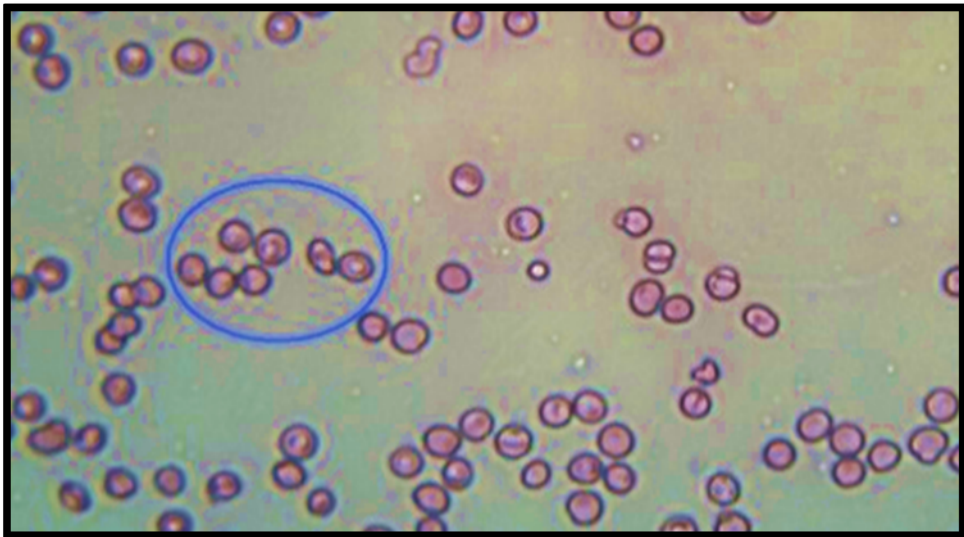


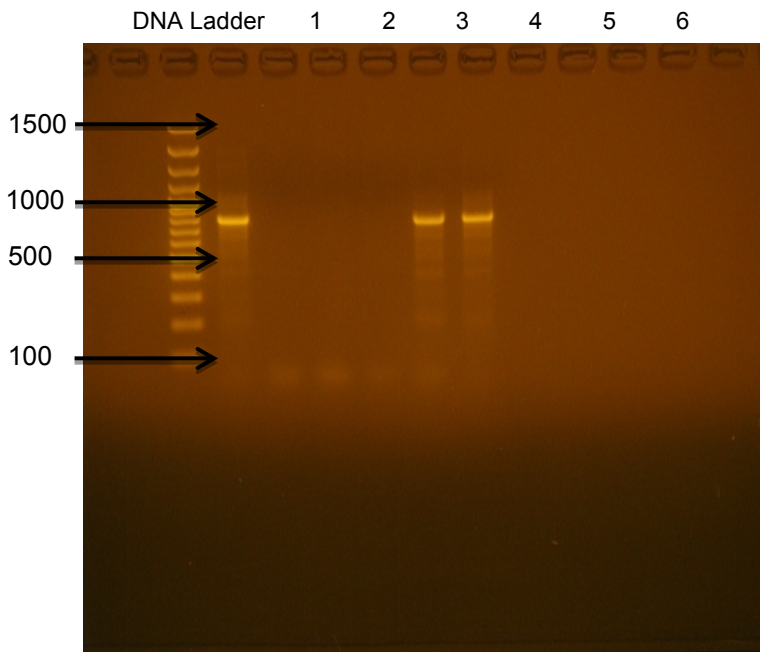
Plate 1. Thin blood smear with multiplying schizonts (x40)

Table 1 shows data regarding malaria. According to data, out of 50 processed samples, 18 (36%) were found positive for plasmodium through blood smear microscopy, whereas when same blood samples were subjected to PCR assay, 6 (12%) samples were found to have genomic DNA of plasmodium. Table 1 further reveals that out of 50 samples from malaria suspected victims, 30 samples were collected from Tandojam out of 30, only 8 (26.6%) were found positive via microscopy, whereas when these samples were subjected to PCR protocols, only 1 sample (3.33%) was found positive having genomic DNA of

Plasmodium. This table further depicts that, out of 20 samples collected from Umerkot, 10 (50%) were positive via microscopy, whereas when same samples were subjected to PCR, 5 (25%) were found positive carrying Plasmodium genomic DNA. This table clearly shows that, diagnosis through microscope may not be relied upon, because PCR based assay does not confirm the presence of the plasmodium parasite. During present study; blood samples processed by routine microscopy were subjected to amplification of genomic DNA by PCR. The PCR results are presented in Plate 2 which show presence of genomic DNA bands at 850 base pairs. According to published primers, genus plasmodium was found to be present in the blood samples.

Table 1. Detection of malaria by microscopy and PCR

Location	Total samples	No (%) positive by microscopy	No (%) positive via PCR
Tandojam	30	8 (26.6%)	1 (3.33%)
Umerkot	20	10 (50%)	5 (25%)
Total	50	18 (36%)	6 (12%)



DNA-Ladder, 1-Pos-Control, 2-Neg-Cont, 3, 4-Negative, 5, 6 Positive

Plate 2. PCR amplification product obtained from blood samples of patients having malarial symptoms, DNA ladder of 100 base pairs, 1 positive control, 2 negative control, 3, 4, negative for Plasmodium DNA, 5, 6 positives for Plasmodium DNA, PCR product indicating DNA bands at 850 base pairs

During this study, one of the aims was to identify the species of plasmodium. With that view, restriction enzymes Alul were used to record the RFLP. According to Plate-3, all plasmodium positive samples were found infected with *Plasmodium falciparum*.

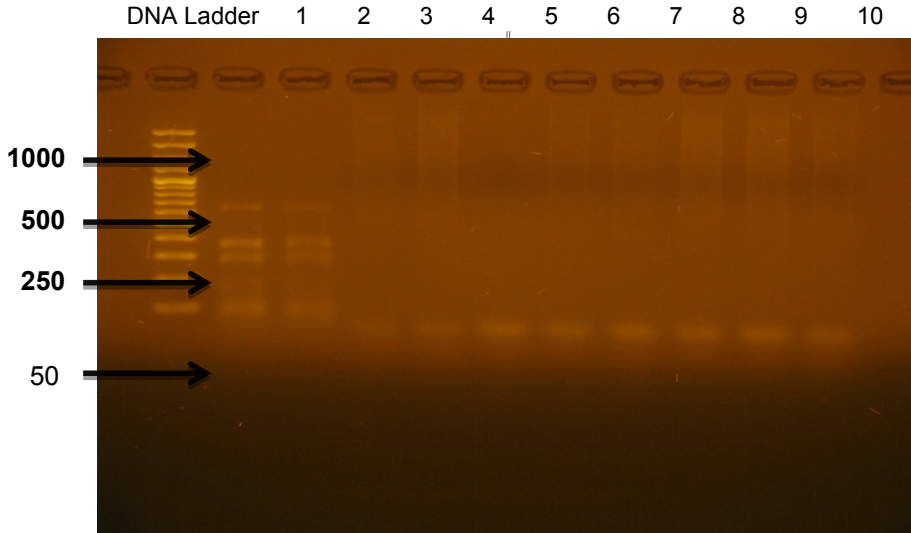


Plate 3. Alul restriction fragment obtained from a PCR product amplified from patients infected with *P. falciparum* bands at 150,180 and 350 bps, DNA ladder 50 base pairs, 1 and 2 digested PCR products and 3-10 undigested PCR products

DISCUSSION

Malaria is still a burning medical problem around the globe. Although substantial progress has been made towards the World Health Assembly target of reducing the malaria burden by 75% by 2015, and the Roll Back Malaria (RBM) target of reducing deaths to near zero (W.H.O.. 2015) yet there is more need to focus on malaria control. The present study was aimed to detect malaria through conventional and molecular techniques from human 50 blood samples were collected from Tandojam, Umerkot, and adjoining areas, further, species of genus plasmodium was identified using Restriction Fragment Length Polymorphism. Another aim of this study was to compare the sensitivity of PCR assay with microscopy.

In this study, infection rate in malaria suspected humans was 36% detected through microscopy and 12% through PCR. Whereas when comparing both techniques 66.60% cases were false positive through microscopy, PCR assay did not confirm these results. Our findings are in agreement with Gonul *et al.* (2007) who used PCR method to determine the presence of plasmodium DNA in blood. They processed 114 positive blood samples and prepared thin and thick blood smears and also subjected samples to extraction of DNA. Extracted DNA was amplified by PCR and the product was digested by restriction enzyme Alul.

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. They also concluded that, as compared to microscopy, the sensitivity, specificity by PCR was more or less perfect.

Blood Smear Microscopy is a widely used diagnostic tool in malaria diagnosis (Singh, 1997) and is still widely used for accurate diagnosis as there are some shortcomings associated with it. In order to overcome some of these shortcomings, molecular or nucleic acid-based diagnostic methods for parasitic infections have been developed and are in use since more than one decade.

In past, there were quite a few advance level diagnostic assays including hybridization of specific probes, to target deoxyribonucleic acid (DNA), but these assays have been replaced by PCR-based assays. For species diagnosis PCR-restriction fragment length polymorphism assays is considered as gold standard and is widely used in epidemiological studies of parasites for being sensitive in detection of pathogen genetic material (Thomas and Martin, 2002).

CONCLUSION

We, like many others conclude that, PCR is highly reliable diagnostic tool and RFLP is certainly best for identification of species. In country like Pakistan, conventional method is mostly used one, whereas, we found issues associated with proper diagnosis, hence PCR protocol is recommended for accurate diagnosis.

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