MOLECULAR METHODS FOR DIAGNOSIS OF MALARIA

Snehanshu Shukla¹, Harshita Pant².

HOW TO CITE THIS ARTICLE:

Snehanshu Shukla, Harshita Pant. "Molecular methods for diagnosis of malaria". Journal of Evolution of Medical and Dental Sciences 2013; Vol2, Issue 28, July 15; Page: 5194-5205.

ABSTRACT: In PCR based techniques specific regions of DNA are amplified, enzymatically through successive cycles, resulting in million-fold amplification of the target sequence of DNA. PCR can detect cases declared negative by microscopy and mixed infection due to its high sensitivity at low level of Parasitemia (F. Perandin et al., 2004¹; Gabriella A. Farcas et al., 2004²; Stephanine P. Johnston et al., 2006³; Monar Ndao et al., 2004⁴).

KEY WORDS: PCR, *P. falciparum*, *P. vivax*, RDT, malaria, SS rRNA.

INTRODUCTION: Molecular method can be used as a research tool to perform quality control checks on microscopic diagnosis of malaria. (Brown et al., 1992⁵, Snounou et al., 1993⁶) or to determine the distribution of important genes associated with drug resistance (Klement E et al., 2001⁷, Ciceron et al., 1999⁸, Humar et al., 1997⁹). The limitation of the PCR technique includes considerable time required for processing, requirement of experience, instrumentation and technical expertise (Robert H. Barker et al., 1994¹⁰). Selection of appropriate primers, method used for collection and storage of samples and extraction methods can all affect PCR performance. In spite of this increasing PCR is being coated as the "new gold standard" for diagnosis of malaria (Haditsch, 2004¹¹; Ndao et al., 2004⁴).

Molecular Diagnostic Assay

In 1984 Franzen et al.¹² reported the isolation and characterization of a repetitive DNA probe to identify *P. falciparum* infected blood in a spot hybridization assay. The identified recombinant clone contained a 21bp tandemly repeated sequences that was specific for *P. falciparum*. Further studies using it found to be very specific for *P. falciparum* but sensitivities parallel that of microscopic examination only in best circumstances. Difficulties in the detection of low Parasitemia in partially immune individuals from malaria endemic areas were particularly noted, as is the situation of India. An effort focused on the detection of plasmodial ribosomal RNA provided sensitivity significantly better than that of repeat-based DNA probes.

PCR

PCR based assays represent a major advance for the laboratory diagnosis of malaria and also have a variety of other research applications (Table R-1).

Advantages of PCR

1. Ability to detect as few as 1-5 parasites/ μ l of blood (<0.001% of infected red blood cells).
2. Large number of samples can be processed at the same time
3. Ability to detect mixed species infections

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Table R-1: Applications of molecular amplification methods for the diagnosis and management of malaria

<ul style="list-style-type: none"> • Clinical situations: • Subpotent Parasitemia associated with chemoprophylaxis or diagnosed during the convalescent period. • Diagnosis of mixed infections • Therapeutic monitoring and prediction of treatment failure • Research settings • Prevalence studies in mainly asymptomatic populations • Antimalarial efficacy trials • Vaccine trials • Reference standard for the evaluation of other non-microscopy methods (e.g. immunochromatographic assays) • Genotype characterization and molecular epidemiology

The sequence of the oligonucleotides used in majority of the PCR assays is based on the small subunit rRNA gene of plasmodium species that infect human (McCutchan et al., 1988¹³). The PCR protocol based on the other oligonucleotide sequences are described in TableR-2:

Table R-2: Examples of Different protocols used for PCR assays.

Amplification Method	Target used for detection and differentiation of Plasmodium species	Used by
Single step PCR	Small subunit ribosomal RNA gene (SS rRNA gene)	Ciceron et al. (1999) ⁸ Das et al. (1995) ¹⁴ Humar et al. (1997) ⁹ Kawamoto et al. (1996) ¹⁵ Schindler et al., (2001) ¹⁶ Witney et al. (2001) ¹⁷
	K1-14 gene	Zhong et al. (1999) ¹⁸
	Circumsporozoite protein gene	Serhabur et al. (1992) ¹⁹
	Erythrocyte binding antigen (EBA-175)	Kain et al. (1991) ²⁰
Nested PCR	Merozoite surface antigen (MSA) gene	Edoh et al. (1997) ²¹
	Erythrocyte binding antigen (EBA-175)	Eoley et al. (1992) ²² Kain et al. (1991) ²⁰
	SS rRNA gene	Ciceron et al. (1999) ⁸ Kawamoto et al. (1996) ¹⁵ Schindler et al., (2001) ¹⁶ Witney et al. (2001) ¹⁷
Multiplex PCR	SS rRNA gene	Schindler et al. (2001) ¹⁶
	Mitochondrial Cox-I gene	Tham J et al. (1999) ²³
Reverse Transcriptase PCR	SS rRNA	Li J et al. (1995) ²⁴ Abdullah et al. (1996) ²⁵ Ciceron et al. (1999) ⁸

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Table R-3: Comparison of some studies using pcr for diagnosis of malaria

Author/ Year of publication/ Place	Primer/Region coding for	Ref. Study method	Sensi-tivity	Speci-ficity	Result	Population	Implication
Nandwani S, Mathur M <i>et al</i> ²⁶ . Delhi, India	A set of primers for MSP1, block 17 (merozoite surface protein) region of <i>P. falciparum</i>	Microscopy	96.8%	100%	310 patients total, Pf positive 160.	India endemic, clinically suspected patients of malaria coming to the malaria centre, Delhi	PCR technique with new modifications offers increased sensitivity and specificity which may be significant in direct identification of drug resistant strains of parasites. It is also useful for other epidemiological studies, detection of mixed infections, low level parasitaemia and evaluation of chemotherapy and vaccine trials.
Kayla F, Laserson <i>et al</i> ²⁷ , (1994), Venezuela	5 different sets of oligonucleotide primers previously described by Barker RH Jr. <i>et al</i> , 1992 ¹⁰ . MSA-1 primer from block-2 repeat region of MSA-1 while MSA-2 primers were from central variable region of MSA-2	PCR	78%	97%	229 samples from 48 patients. The PPV/NPV of PCR methods 8/95.	Venezuela endemic, hospital based field trial.	DNA probe/PCR technology can be useful tool for large-scale epidemiological survey, as opposed to diagnosis of acute malaria in the settings of malaria clinics.
Tirasophon W. <i>et al</i> ²⁸ , 1994, Deptt. of Biochemistry, Mahidol University, Bangkok, Thailand	Pf1 and Pf2 primer for <i>P. falciparum</i> (206bp product) PV1 and PV2 primers (183 bp product) (Rajkulchai P.)	Microscopy	Pf 89% Pv 91%	94% (overall)	590 individuals. PCR could detect 6 mix infections that were characterized to be single species infection by microscopy.	Thailand, endemic, patients presenting to malaria clinic for smear examination.	A highly sensitive, rapid (4 hr) and simple method: So can be used for large scale epidemiological, follow up drug therapy, and immunization trials.
J.M.Rubio <i>et al</i> ²⁹ , 1999, Madrid, Spain.	Semi nested PCR, using four species specific primers for all the malaria species, targeting 18S small subunit rRNA gene.	Microscopy.	100	100	192 whole blood and 71 serum sample. Pf/Pm/Pv/Po, 68%/29%/14%/7%	Immigrants from endemic area	Seminested PCR can be a sensitive and specific tool for malaria diagnosis in emigrants from disease endemic to non-endemic region
Geoffrey <i>et al</i> ³⁰ , 2002. Westmead, Australia	Seminested, multiplex PCR; using primers targeting 18S small subunit rRNA gene of plasmodium as previously described by Rubio <i>et al</i> .	NA	ICT (Pf/Pv) 97%/85% Optimal assay: Pf/Pv, 85%/80%	90%/100% for Pf/Pv by ICT and 96%/97% for Pf/Pv by OptiMAL	158 specimen from 144 patients. Positive 93 specimen of 87 patient. 3 additional positive by PCR.	Febrile returned travelers from malaria endemic regions.	Despite the high sensitivity of the ICT p.f/p.v assay for <i>P. falciparum</i> malaria, caution is warranted before RDT's are widely adopted for the diagnosis of malaria in non-immune patients or in countries where malaria is not endemic.
Russel E	Nested PCR using primers	Microscopy	96%	98%	Total 8590 samples,	Thailand endemic	PCR can be used during

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Author/ Year of publication/ Place	Primer/Region coding for	Ref. Study method	Sensi-tivity	Speci-ficity	Result	Population	Implication
Coleman <i>et al</i> ³¹ . (2002), Bangkok, Thailand	described by Kimura <i>et al</i> . ³² from the small subunit ribosomal RNA gene				665 positive Pf/Pv, 265/386	Field based clinical trial.	active malaria surveillance for malaria diagnosis though its sensitivity fell at parasite density <100/microlitre in this study.
E. Patsoula <i>et al</i> ³³ . (2002), Athens, Greece	Simple PCR, using three primers PL3 common to Pf and Pv PL4 specific for Pv, PL5 specific for Pf amplifying a sequence common to small -subunit rRNA gene.	Microscopy	100%	100%	124 blood sample PCR positive 56 (Pf/Pv; 53/3)	Most positive (34 of 56) cases had emigrated to Greece from malaria endemic areas of Africa and Asia. The remaining 22 were Greeks who had travelled to malaria-endemic countries for holidays	A simple, single step PCR can be a faster method which offers good sensitivity and specificity, so a powerful tool for surveillance of migrants or travellers from malaria non-endemic region to malaria endemic regions.
Mathieu Rougemont <i>et al</i> ³⁴ . Institute of Micro-biology, Lausanne, Switzerland	Multiplex RT PCR using block 9 region forward primer Plasmo 1 and reverse primer Plasmo 2 to amplify a 157 to 165 bp segment of the four plasmodial 18S genes. Both primer and probe sequences were selected from the small subunit of rRNA .	Microscopy	All the discardant result with regard to species and genus identified resolved in favour of molecular (PCR/Probe) methods		86 samples. 97 blood samples 86% concordance between PCR and microscopy.	imigrants to epidemic region	A single stage multiplex PCR not only permits a malarial infection to be detected but also allows speciation in cases of mixed infection and monitoring patient on antimalarial therapy.
F. Perandin <i>et al</i> ¹ . (2004), Pasteur Institute, Paris, France.	Nested PCR using previously used genus specific primers by Snounou <i>et al</i> . ⁶ RTPCR for Pf, Pv and Po using sequence quoted by Snounou <i>et al</i> . ⁶ to specifically amplify 18S rRNA gene	Nested PCR	100%	100%	122 whole blood sample, retrospective study, 60 positive, 60 negative. All the mixed infection and correct species identification were done by RT PCR	Travellers to endemic regions	RT PCR picked up mixed infection and it is faster, sensitive alternative to nested PCR and minimizes risk of contamination in post PCR processing so can be implemented in routine diagnostic malaria test once further studies confirm clinical value of this technique.
Gabriella A. Farcas <i>et al</i> ² . (2004); Toronto, Canada	RT PCR, using primers designed for 18S rRNA gene ,for all malaria species	Nested PCR	99.5%	100%	259 febrile individuals .Positive, Pf/Pv: 101/90; Po/Pm : 9/3; 6 mixed infection, 50 negative	Canada, travelers from endemic regions.	RT PCR is rapid (245 min), sensitive and specific method for detection of malaria in returned travelers.
Momar Ndao <i>et al</i> ⁴ . (2004), Quebec, Canada	Nested PCR using primers designed by Snounou <i>et al</i> . for 18S ribosomal subunit of all four malaria parasite	Nested PCR	50% Microscopy 37.5% ICT 29.1%	100% Microscopy 100% ICT 95.6%	Positive 98 of 521 out of which 81 are Pf rest other species.	Refugee in Quebec and Canada from Tanzania Camp	PCR is by far the most powerful tool for surveillance of migrant population and mitigate against transmission to the host population.

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Author/ Year of publication/ Place	Primer/Region coding for	Ref. Study method	Sensi-tivity	Speci-ficity	Result	Population	Implication
			Optimal	Optimal			
Stephanine P. Johnston <i>et al</i> ³ , (2006), Atlanta, Georgia	Nested PCR assay, using modification of technique described by Snounou <i>et al</i> ⁶ , with primers targeting all the Plasmodium species 18S rRNA genes	Nested PCR (Snowon <i>et al</i> ⁶)	93.3%	100%	176 sets of stained blood films received at the CDC for routine or confirmatory malaria diagnosis. PCR detects of specimens that were Negative on microscopy Mixed infection in 5 samples out of which only 1 was resolved by microscopy.	Travellers to malaria endemic regions	Nested PCR was more sensitive than microscopy, allowing <i>P.falciparum</i> detection in cases of low parasitemias, as well as mixed infections .
S. Gatti <i>et al</i> ³⁵ , 2007 Laboratory of Parasitology, Virology, Pavia, Italy	Conventional PCR using five conventional kits. AMS61, AMS42, AMS43, AMS44, AMS45, Clorit sub Milano Italy based on 18S ribosomal RNA gene of parasite for different plasmodium species. AM61 is panspecific (other than <i>P. falciparum</i>) while rest are species specific kits.	Microscopy	ICT – Now malaria 100% for Pf AMS61 Assay 91.0% for all malarial infection	100% for Pf 98.2% all malarial infection	306 subjects: 171 Italians and 135 non-European.	European and Italian migrants from malaria endemic countries.	Compared with the microscopy both ICT and PCR-based assays were poor at identifying mixed infections.
Dhanpat K. Kochar <i>et al</i> ³⁶ , 2009. Dept. of Medicine S.P. Medical College, Bikaner, Rajasthan, India	Primers for 18S ribosomal RNA gene of Plasmodium and used, 1 genus specific 5' primer, 2 species specific 3' primer in same reaction mixture.	Microscopy	N.A.	NA	1091 patients 635/456; Pf/Pv	India, endemic, adult clinically suspected patients of malaria admitted to medicine ward of the study hospital included.	PCR as a useful tool to diagnose severe <i>P.vivax</i> malaria.

Abstracts of some studies on comparing pcr with microscopy and rapid diagnostic tests (rdt) suggesting advantages and limitations

In a study titled “Limited level of accuracy provided by available rapid diagnosis tests for malaria enhances the need for PCR-based reference laboratories” conducted by JM. Rubio *et al*³⁷., in 2001, at Department of Microbiology and Department of Infectious Diseases, Madrid, Spain .The author’s data suggest that the accuracy of RDT's is insufficient and could increase the number of incorrect malaria diagnoses. RDT's could help the initial assessment of malaria in returned travellers and migrants, but there is a need to develop more specialized laboratories with available

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confirmatory diagnostic techniques (PCR). The main difficulty still encountered by the use of RDT's is the correct identification of plasmodium species.

In another study in Department of Medicine, Toronto Canada David C. Richardson, et al³⁸. evaluated the performance of one rapid non-microscopic antigen detection assay, the Makromed dipstick test, blindly compared to PCR and microscopy for the diagnosis of *Plasmodium falciparum* malaria in 200 febrile returned travellers. The Makromed assay detects the presence of *P. falciparum*-specific histidine-rich protein II with an antigen capture immunochromatographic strip format. Compared to PCR as the reference standard, the dipstick assay had a sensitivity of 97.0% and a specificity of 96.0%. The positive and negative predictive values were 81.2% and 99.5%, respectively (Journal of Clinical Microbiology, Dec. 2002, p. 4528–4530).

In a study conducted by Jamshaid Iqbal et al³⁹., in 2002 in Department of Microbiology, Faculty of Medicine, Kuwait University, and Malaria Laboratory, Department of Community Health, Ministry of Health, Safat, Kuwait titled “Comparison of the OptiMAL test with PCR for the detection of *Plasmodium falciparum* and *Plasmodium vivax* infection diagnosis of malaria in immigrants from endemic tropical countries” using microscopy and a PCR as reference standards in 550 immigrants who presented with fever. The PCR and species identification were performed as described by Hang et al⁴⁰. with slight modifications. Oligonucleotide primers which amplify a 206-bp sequence were used for *P. falciparum*, and primers which amplify a 131-bp sequence from the gene for the small subunit of rRNA were used for *P. vivax*. The study concluded that the sensitivity and specificity of the optimal test are comparable to those of microscopy in detecting malaria infection at a parasitemia level of >100 parasites/ml; however, the test failed to identify more than half of the patients with a Parasitemia level of <50 parasites/ml. Thus, the optimal test should be used with great caution, and it should not replace conventional microscopy in the diagnosis of malaria infection.

Kathleen J. et al⁴¹., in a study titled, “Evaluation of a colorimetric PCR-based assay to diagnose *Plasmodium falciparum* malaria in travelers”, conducted in tropical disease unit, division of infectious diseases, Department of Medicine, Toronto Hospital, Canada. The authors performed a blinded evaluation of a non isotopic colorimetric PCR based assay (Digene sharp signal system) and compared the results with those obtained by microscopy and nested PCR for the detection of the *P. falciparum* malaria in 150 febrile travelers. Patients presenting to the tropical disease unit of the Toronto Hospital from June 1995 to July 1997. A nested PCR method for the amplification of a fragment of the plasmodial small-subunit rRNA gene was performed as described previously by Snounou et al.⁶. The sensitivity and specificity of the Digene kit for the diagnosis of *Plasmodium falciparum* infection were calculated by using nested PCR-based species identification as the reference standard. The colorimetric assay had a sensitivity of 100% and a specificity of 95.4% for the detection of *Plasmodium falciparum*. This PCR based non-isotopic assay is a rapid, sensitive, and specific method for the detection of *P. falciparum* malaria in returned travelers. It may be particularly useful for the identification of *P. falciparum* in patients with mixed infections or in patients with low circulating levels of parasitemia, for whom reliable species determination by microscopy is not always possible. A limitation of this test is that only *falciparum* malaria is detected; however, it may be combined with a similar assay for the diagnosis of *P. vivax* malaria.

A study titled “Development of a PCR assay followed by nonradioactive hybridization using oligonucleotides covalently bound to Covalink NH microwell for detection of four plasmodium

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species in blood samples from humans” published in journal of clinical microbiology, Sept. 2006 conducted by M. Machouart et al⁴². The authors developed and evaluated a PCR-based assay to detect four *Plasmodium* species in 79 blood samples from 56 travelers returning from areas where malaria is endemic (for some patients, several blood samples were collected during the first days of the hospitalization period) and presenting suggestive clinical features were collected in parasitology-mycology laboratories of hospitals in Nancy and Tourcoing (France). DNA amplification targeting a small region of the 18S-rRNA gene was performed with *Plasmodium* genus-specific primers. The biotinylated PCR products were then identified by PCR-colorimetric Covalink NH microwell plate hybridization (CMPH), using species-specific phosphorylated probes covalently bound to a pretreated polystyrene surface. The results from PCR-CMPH showed high specificity, and for 47 of the 56 patients (84%), microscopy and PCR-CMPH results were in agreement. Discordant results were reevaluated with microscopy examination, other molecular methods, and DNA sequencing. Except for one patient, discrepancies were resolved in favor of PCR-CMPH. Three mixed infections were detected, four species identification errors were corrected and two negative results were shown to be positive. The study results indicate that PCR-CMPH is a simple, rapid, and specific method for malaria diagnosis. It employs stable reagents and inexpensive equipment, making it suitable for routine epidemiological use. (Journal of Clinical Microbiology, Sept. 2006, p. 3279–3284 Vol. 44, No. 9).

In a study titled “Real-Time PCR for detection and identification of *Plasmodium* spp.”, conducted by Kathy A. Mangold et al⁴³, at Department of Pathology and Laboratory Medicine, Evanston Northwestern Health-Care, Evanston, published in Journal of Clinical Microbiology, May 2005, the authors developed and used a Real-Time PCR assay to detect and distinguish four *Plasmodium* spp that cause human disease, by using a single amplification reaction and melting curve analysis. For assay validation, 358 patient blood samples from the national university hospital in Singapore and Evanston Northwestern Healthcare in Illinois were analyzed. Consensus primers were used to amplify a species-specific region of the multicopy 18S rRNA gene, and SYBR green was used for detection in a light-cycler instrument. Patient specimens infected at 0.01 to 0.02% parasitemia densities were detected, and analytical sensitivity was estimated to be 0.2 genome equivalent per reaction. Melting curve analysis based on nucleotide variations within the amplicons provided a basis for accurate differentiation of *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Of 76 blinded patient samples with a microscopic diagnosis of *P. falciparum*, *P. vivax*, or *P. ovale* infection, 74 (97.4%) were detected by Real time PCR including three specimens containing mixed *P. falciparum*-*P. vivax* infections. Real-time PCR with melting curve analysis could be a rapid and objective supplement to the examination of Giemsa-stained blood smears and may replace microscopy following further validation.

In a study titled “Evaluation of Real-Time PCR FRET assay for rapid detection and differentiation of plasmodium species in returning traveller’s and migrants” conducted by Innocent Safeukui et al⁴⁴. at travel clinic and imported diseases units, department of internal medicine, university hospital centre, France published in Malaria journal 2008, the authors collected a total of 119 blood samples in EDTA tube from traveller’s or migrants admitted to the Saint André university hospital in Bordeaux, with suspected clinical malaria from years 2000 to 2006. The primers and probes were designed using the multicopy, stable and highly conserved 18S rRNA gene single-stranded sequences of plasmodium species available from genbank. The sequence of the

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acceptor FRET hybridization probe was designed on the basis of one nucleotide mismatch difference that distinguish the 18S rRNA gene of the *P. falciparum* from those of the three other plasmodium species. The *P. falciparum* specific primers were designed to amplify a 120-bp region from the cytochrome c-oxidase subunit 1 (cox-1) mitochondrial gene. A rapid FRET Real time PCR, using one set of primer and probe for the diagnosis of *Plasmodium* sp. febrile returning traveller's and migrants was described. An important advantage of this approach is the higher sensitivity and specificity for the detection of *Plasmodium* sp. and to differentiate *P. falciparum* from the three other malaria parasite species (*P. vivax*, *P. malariae* and *P. ovale*) based on the melting. Concurrently to microscopic examination, such results can be used to ensure rapid treatment administration and proper follow-up of the malaria attacks by the practitioner.

Conclusion: Although there have been many previous publication on the detection of malaria and identification of plasmodium species by PCR, all relates either to the use of some form of nested PCR, using nested or heminested primers (Snounou et al.⁶, 1993a,b; Rubio et al.³⁷, 1999; Gal et al.⁴⁵, 2001), or to the detection of amplified DNA using labeled, specific probes or ELISA (Laoboonchai et al.⁴⁶, 2001), for species identification. Single round PCR has only been used alone to detect a particular species, usually *P. falciparum* (Tham et al.²³, 1999; Barker et al.¹⁰, 1994; Hang et al.⁴⁰, 1995), or simply to detect malarial infection without species identification (Ciceron et al.⁸, 1999). The method is relatively quick, compared to nested and hemi-nested PCR, and can be completed within 3 h of sample receipt (Patsoula E et al.³³, 2003). Thus, a same day diagnosis of malaria could be provided, and the assay would clearly be useful in a reference-laboratory setting at a center where majority of the patients are of complicated malaria, have already taken antimalarial treatment on clinical suspicion prior to being investigated at the referral center laboratory. The risks of cross contamination, which could be a problem with assays based on nested PCR unless suitable, stringent precautions are observed, is relatively low for single-round assays. The test could provide a powerful back-up to the blood smear examination (which is still far from redundant and is not replaced by this technique.) and should prove very useful in detection of parasitaemias which is too low to be detected in blood smears (Babiker et al.⁴⁷, 1999).

ACKNOWLEDGEMENT: This review in part is supported by Dr. Vimala Venkatesh and Dr. Mastan Singh, Post Graduate Department of Microbiology, King George's Medical University, Lucknow, U.P., India.

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AUTHORS:

1. Snehanishu Shukla
2. Harshita Pant

PARTICULARS OF CONTRIBUTORS:

1. Assistant Professor, Department of Microbiology, Mayo Institute of Medical Sciences", Barabanki, Lucknow.
2. Assistant Professor, Department of Radiodiagnosis, Mayo Institute of Medical Sciences", Barabanki, Lucknow.

NAME ADDRESS EMAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Snehanishu Shukla,
Plot-1, Basant vihar Colony,
Behind St. Mary's Inter College, Sect.14,
Indira Nagar, Lucknow, U.P- 226016.
E-mail: shuklasnehanishu@gmail.com

Date of Submission: 01/07/2013.

Date of Peer Review: 01/07/2013.

Date of Acceptance: 03/07/2013.

Date of Publishing: 15/07/2013