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Selecting better diagnostic kits for diagnosis of malarial parasites at point of care

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Abstract

Malaria is a fatal life-threatening parasitic infection and a leading cause of morbidity and mortality. The present study was aimed to evaluate simple, inexpensive, accurate, reliable, easily available better diagnostic for rapid detection of malaria at point of care (POC). The study includes 1403 samples collected from the patients, of which 1227 were clinically suspected cases and 176 from consecutive feverish patients. Among the suspected cases only 338 samples were confirmed positive and 889 samples were negative for *Plasmodium* species by PCR. All the 889 samples showed negative result for *plasmodium* species by microscopy, Malarial Ag rapid kits but only 867 samples were confirmed negative with malarial Ab rapid kits. Of the 338 PCR positive samples, 337 samples were confirmed positive by microscopy and Malarial Ag rapid kits, but only 284 samples were confirmed positive using malarial Ab rapid kits. Overall the microscopy and the malaria antigen-based lateral flow assay exhibited similar sensitivity, specificity, PPV, NPV and efficiency, respectively, whereas the PCR assay had 100% sensitivity, specificity, PPV, NPV and efficiency. But the evolutionary data for malaria antibody lateral flow assay has 92.81% sensitivity, 94.13% specificity, 84.02% PPV, 97.52% NPV and 93.80% efficiency. The developed Malaria *pf/pv* antigen and antibody field-deployable kits are simple, rapid, accurate, reliable, inexpensive, user friendly, POC. In addition the kits are highly sensitive and species-specific. The *pf/pv* antigen kit is found to be more accurate with 99.7% sensitivity and 100% specificity than to Malaria *pf/pv* antibody rapid kits. Of the two rapid kits developed, Malaria *pf/pv* antigen kit is found be more accurate with 99.7% sensitivity and 100% specificity than to Malaria *pf/pv* antibody rapid kits.

Keywords Point of care (POC) · Immuno chromatographic test (ICT) · *Plasmodium Lactate Dehydrogenase* (pLDH) · Histidine Rich Protein II (HRPII) · *Plasmodium falciparum* (pf) · *Plasmodium vivax* (pv) · Antigen (Ag) · Antibody (Ab) · Rapid diagnostic test (RDT) · Polymerase chain reaction (PCR)

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Introduction

Point of care diagnostics (POC) are increasing day by day, due to its cost effectiveness and easy to use in resource-limited areas. There is an emergent demand for POC diagnostics in developing countries (Notomi et al. 2000). The number of deaths occurring in developing countries annually is due to the improper diagnosis despite progress in medical science. Even though development of effective chemotherapeutics has progressed, infectious diseases still continue to affect millions of lives around the world (Cohen 2000). Malaria is one of the most important tropical infectious diseases that cause approximately 216 million cases of malaria that occurred worldwide and 4,45,000 deaths from malaria globally, compared to 4,46,000 estimated deaths in 2015 (WHO World Malaria Report 2017). The disease is endemic around

the equator keeping approximately 50% of world's population at risk. Hence, diagnosis is important for the prescribing of effective drugs and treatment (Urdea et al. 2006). The range of areas inhabited by mosquitoes harbouring malarial parasite is currently expanding due to global climate change (Patz and Olson 2006). So, proper and accurate diagnosis of malaria is extremely important. The existing tools for the diagnosis of malaria include microscopy, parasite Ag and Ab detection rapid test kits and molecular tools (Bronzan et al. 2008).

For more than 100 years, the gold standard method for the diagnosis of malaria is microscopic examination of thick and thin (Sirichanisinthop et al. 2011) Giemsa, wright- or field-stained blood films (Rambabu et al. 2013). However, the microscopy has some limitations: such being time-consuming, and misdiagnosis of the mixed species infection is common if the microscopist lacks experience and even when the parasitemia is low (Mallepaddi et al. 2016). The poor sensitivity of smear tests often causes fatal delay in treatment (Hanscheid and Grobusch 2002; Mallepaddi et al. 2016). The labour-intensive nature of microscopy and the requirement for high levels of operator training have led to the development of several alternative techniques for laboratory diagnosis of malaria in both endemic and non-endemic areas (Spencer et al. 2010). Several molecular assays such as PCR assays have also been developed for the detection and identification of malarial parasites based on genus- and species-specific sequences of the parasites (Hanscheid and Grobusch 2002; Spencer et al. 2010). PCR assay is more specific and sensitive than microscopy (Zhong and Kain 1999; Moody 2002; Rambabu et al. 2013), and as few as 0.7 parasites per microliter of blood can be detected (Chen et al. 2016). Regrettably, the PCR assays are beyond the capacity of most malaria endemic areas as they need very expensive, sophisticated laboratory and well-trained personnel (Rambabu et al. 2013).

So, taking all these resource-limited settings and point of care areas into consideration the lateral flow assay (Ab and Ag rapids kits) is the best diagnostic method directly used at field level or at point of care areas (Beadle et al. 1994; Makler et al. 1998; Moody 2002; Rambabu et al. 2013). These lateral flow methods do not offer improved sensitivity over microscopy when parasitemia fall below 100 parasites per µl (Mills et al. 1999). The drawbacks of this format include its susceptibility to degradation in suboptimal storage conditions, limited shelf life and an apparent failure to detect some parasites is due to mutations within target antigen (Lee et al. 2006), and the possibility of false positive results due to persistence of the antigen (Moody 2002; Bell et al. 2005; Abeku et al. 2008; Bharti et al. 2008; Spencer et al. 2010). Though there might be a problem in the stability, sensitivity and specificity of the lateral flow assays, most of the diagnostic approaches are attracted by lateral flow

test kits only. The lateral flow kits have a greater potential approach because of its simple, easy and inexpensive point of care handling procedures and infrastructure-free settings than the other existing diagnostic methodologies. Due to this reason the lateral flow test kits play a major role in the diagnosis and have become the basis for the most of the commercial diagnostic approaches (Shiff et al. 1993; Piper et al. 1999; Mallepaddi et al. 2016). The present study was aimed to develop Malaria *pf*/*pv* antigen and antibody rapid kits and screening of 1403 samples using the Malaria *pf*/*pv* antigen and antibody rapid kits feild deployable. In addition the sensitivity and specificity of Malaria *pf*/*pv* antigen and Malaria *pf*/*pv* antibody rapid kits were compared with the gold standard microscopy and PCR results.

Materials and methods

Clinical study site

The complete clinical study and the collection of the samples were performed at Department of Research, MNR Medical College during the period of three years from March 2014 to May 2017 and development, validation and diagnostic studies were carried out at Genomix Molecular Diagnostics Pvt.Ltd, Hyderabad, India. The study group included 1227 clinically suspected samples. This study was approved by the Institutional Ethical committee. Informed consent was taken from all those patients with suggestion of malaria infection, who were included in this study. Informed consent forms (questionnaire in Telugu and English languages) were provided.

Collection of samples

The whole blood of the each individual was collected in a vaccutainer that is pre-treated with trace amount of EDTA. The collected samples were examined by microscopy followed by PCR analysis and the remaining sample was plotted on a filter paper and stored at − 20 °C for further analysis (Rambabu et al. 2013; Mallepaddi et al. 2016).

Microscopy

Microscopy is the gold standard method used for the specific detection of malaria parasites using thick and thin blood films stained using Giemsa stain method (Okell et al. 2012). The thick smear helps in the identification of malarial parasites to the genus level, whereas the thin smear helps in species level identification of the malarial parasites (Notomi et al. 2000; Moody 2002; Rambabu et al. 2013).

Lateral flow immune chromatographic test kits

Immuno chromatography or lateral flow diagnostic test kits work on the principle of chromatography of antigen and antibodies. The facilities at Genomix Molecular Diagnostics Pvt Ltd, Hyderabad have been used for manufacturing of Malarial Pf/*Pv* Ag and *Pf/Pv* Ab rapid diagnostic test kits and collected blood specimens were diagnosed using antigen and antibody lateral flow diagnostic test kits. The diagnosis was based on the HRP II (histidine rich protein II) specific antigenic protein for *Plasmodium falciparum* (*pf*) and pLDH (*Plasmodium* lactate dehydrogenase) antigenic protein specific for *Plasmodium vivax* (*pv*) of the malarial species.

Materials used for lateral flow kits preparation

The materials used for development of lateral flow test kits include the following components: sample pad, nitrocellulose membrane, absorbent pad, supporting matrix and conjugate matrix procured commercially (MDI membranes, Ambala, India). The lateral flow components such as monoclonal and polyclonal antibodies specific for *Plasmodium vivax, Plasmodium falciparum* malarial parasites were developed, purified and characterized for making the malaria antigen lateral flow test kits, whereas the purified KLH (Keyhole limpet hemocyanin) conjugated peptide antigenic proteins were procured commercially (*Genemed Synthesis Inc, USA*) specific for *Plasmodium vivax* strain Gwalior lactate dehydrogenase (pLDH) with GenBank Accession No EU262983.1 and *Plasmodium falciparum* strain 3D7 Histidine-rich protein II gene, with GeneBank Accession No AL844507.3 with locus tag PF3D7_0831800 were used to make the lateral flow test kits.

Preparation of polyclonal antibodies

The synthetic peptides pLDH of *plasmodium vivax* and HRP II of *plasmodium falciparum* were mixed with Immunoblast adjuvant along with 0.1M phosphate buffer saline and immunized to goats (Lee et al. 2011). A total of 200 µl volume of synthetic peptide adjuvant mixture were immunized to goats and boosters were given at 15 days' time interval. The immunized boosters were collected and purified using affinity chromatography with protein G Sepharose (Thermo Fisher Scientific, USA). The purified goat IgG antibodies pLDH-specific 10G9.2E7 of *plasmodium vivax* and HRPII-specific polyclonal goat IgG 2A5.2C3 of *plasmodium falciparum* were further dialysed into 0.1M phosphate buffer saline and the concentration of purified antibodies was brought to 1.0 mg/ml by pooling all the eluted fractions followed by lyophilisation process.

Finally, the purified goat IgG antibodies against pLDH (10G9.2E7) and HRP II (2A5.2C3) with a concentration of 1 mg/ml were used at T1 and T2 positions on the malaria *pf/pv* Ag rapid test kit.

Preparation of monoclonal antibodies

The synthetic peptides of *plasmodium vivax* (pLDH) and *plasmodium falciparum* (HRP II) antigens were used to immunize the 7-week-old female BALB/c mice individually. The synthetic peptides were mixed with Immunoblast adjuvant and immunized at a volume of 200 µl as a first immunization followed the same volume for two more times with 14-day time interval. The antibody titer developed against these two synthetic peptide antigens pLDH and HRP II was cross checked using an enzyme-linked immunosorbent assay in a multiwall plate coated with 1 mg/mL of these synthetic peptides individually. The optical density was read at OD 450 of a 1000-fold dilution of blood. The next booster immunization step was performed once the optical density was greater than 0.3, by injecting 200 µl of mix of adjuvant with synthetic peptide into the tail vein of the respective mice. On the third day after the process of final booster of immunization, the Sp2/0 myeloma cells were fused with mouse spleen cells resulting in the hybridomas' production as described using Kohler and Milstein (1975), Lee et al. (2011) protocols. The specific hybridoma clones 10G9.2G5 (*Plasmodium vivax* pLDH) and 3E12.1D4 (*Plasmodium falciparum* HRP II) produced were positive and were selected for sub cloning using dilution method. The screened hybridomas were further used to produce the ascitic fluid in the mice. The IgG antibody was purified using protein G Sepharose column chromatography. The concentration of eluted fractions was estimated using nano drop 1000 instrument and stored at −20 °C for further use.

Preparation of gold particles

The colloidal gold particles with 40 nm size were prepared using the modified in-house protocol using Horisberger and Clerc (1985) study. In the preparation of colloidal gold solution 100 ml of 0.01% aqueous gold solution (HAucl$_4$) was boiled on a magnetic stirrer with continuous heat. A sum of 1.6 ml of 1% tri sodium citrate was added drop by drop to the aqueous gold solution pursued by the shading change from purple to dark red with persistent mixing with boiling (Lee et al. 2011). After adding the citrate, the colour change from black to purple and deep red was noticed. After the colour change to deep red, the solution was allowed to cool to room temperature. The cooled colloidal gold solution was transferred on to a glass bottle and stored at 4 °C for further use.

Conjugation of monoclonal antibodies with gold particles

The purified monoclonal antibodies that were raised against the synthetic peptide antigens pLDH-specific for *plasmodium vivax* and HRP II-specific for *plasmodium falciparum* were conjugated with colloidal gold particles using the in-house modified protocols from Cramer et al. 1989. The purified monoclonal antibodies 10G9.2G5 of *plasmodium vivax* (pLDH) and 3E12.1D4 of *plasmodium falciparum* (HRP II) were dialysed into 0.1M phosphate buffer saline overnight at 4 °C with continuous stirring with magnetic stirrer. The pH of the prepared colloidal gold solution was adjusted to pH 9.0 with 0.1 M HEPES solution and mixed with 4 μg/ml concentration of purified and dialysed fractions of monoclonal antibodies. The complete solution was allowed to incubate at room temperature for 1 h on a rocker. After the process of incubation 2% bovine serum albumin was added to stabilize the conjugated antibodies solution (Horisberger and Clerc 1985; Lee et al. 2011). The conjugated antibody gold solution was centrifuged at 6000 rpm in a cold ultra centrifuge for 15 min at 4 °C. The gold particles were settled at the bottom of the tube as a loose pellet. The supernatant was removed without disturbing the pellet and reconstituted with 0.1M phosphate buffer saline with pH 8.0. The process was repeated twice to remove the unbound antibodies in the solution. Finally, the gold-conjugated antibody pellet was resuspended in phosphate-buffered saline containing 1% bovine serum albumin and the absorbance was measured at 450 nm.

Lateral flow kit preparation process

Development of malaria antigen lateral flow kit

The malarial *Pf/Pv* Ag lateral flow test kits were developed using the polyclonal and monoclonal antibodies developed against the pLDH and HRP II synthetic peptides of *Plasmodium vivax* and *Plasmodium falciparum*. The polyclonal goat IgG antibodies pLDH-specific 10G9.2E7 for *plasmodium vivax* and HRPII-specific polyclonal goat IgG 2A5.2C3 of *plasmodium falciparum* with 1 mg/ml concentration were coated on the test line T1 position and T2 position, respectively, with biotin at the "C" control position on a nitrocellulose membrane using the Bio Dot Quanti-2000 Biojet

coating machine. The gold nano particles were conjugated with disease-specific monoclonal antibodies 10G9.2G5 (*plasmodium vivax* pLDH) and 3E12.1D4 (*plasmodium falciparum* HRP II) along with streptavidin. The gold-conjugated solution was dispensed on to a conjugate matrix and air dried in a dehumidified room with relative humidity less than 20%. One side of the nitrocellulose membrane was overlapped with absorbent pad and the other end was overlapped with gold conjugate pad followed by sample pad. All the membrane pads overlapped partially to ensure the continuous flow of sample along test strip. The in-house sample diluent buffer was used while testing the kits for lysis RBC. The diluent contained 0.2% Tris, 2% casein, 1% triton-X and the pH should be maintained in between 8.0 and 9.0.

Development of antibody-based lateral flow kit

The malaria species-specific antibody lateral flow test kits were developed with the synthetic antigenic peptides (*Pf/Pv*) that were specific for *Plasmodium vivax* and *Plasmodium falciparum* which were coated on the test line (T1 and T2) positions along with biotin and the control line position of the nitrocellulose membrane. *Pf/Pv* specific peptide proteins were gold conjugated along with streptavidin and were dispensed on the conjugate matrix and were dried in a dehumidified room. The in-house sample diluent buffer was used while testing the kits for lysis of the RBC. The diluent contains 0.2% Tris, 2% casein, 1% triton-X and the pH should be maintained in between 8.0 and 9.0.

Lateral flow assay procedure

As per the protocol developed the collected samples were analysed using these lateral flow test kits. A finger prick whole blood or 5 μl of collected whole blood specimen on the filter paper was directly added to the sample well using the sample holder dropper followed by addition of two drops of sample diluent (Fig. 1). After the addition of sample diluent the sample along with diluent tends to move towards the absorption pad by capillary action on nitrocellulose membrane.

Fig. 1 Diagrammatic representation of test process of Malaria Lateral flow assay
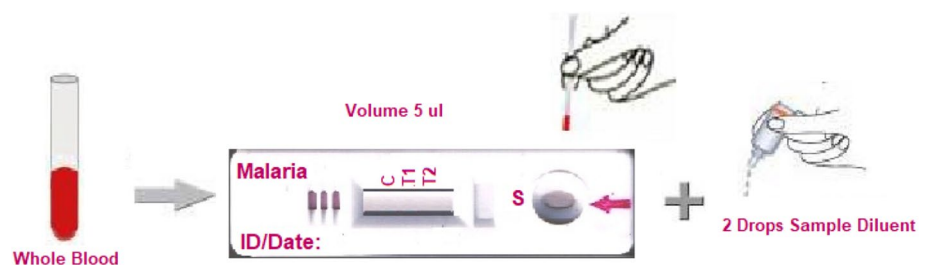
Analysis of the lateral flow assay

The lateral flow test kit results were analysed by observing the clear purple coloured bands that are visible to our naked eye on the results window of the test cassette. If coloured bands appear at control and Test (T1) positions, it means that the sample was positive for *P. vivax*, whereas the coloured lines at control and Test (T1 and T2) positions indicate that samples were positive for both the *P. vivax* and *P. falciparum*. If the coloured lines appeared at control and test (T2) positions then the sample is positive for *P. falciparum* only. The coloured band at control line alone denotes that the sample is negative for malarial parasites as shown in Fig. 2. If in the absence of control coloured lines formed at T1 and T2 or there is no line at results window, then the assay was invalid and it was recommended to repeat with new kit.

Statistical analysis and data management

The collected whole blood samples were tested with both the antigen (Ag) and antibody (Ab) lateral flow test kits. Calculation of specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) was carried using the statistical analysis software SPSS20.0 (Joseph and Fleis 2003). The statistical analysis for these samples was performed using PRISM (Graph Pad Software, San Diego, CA).

Results

Out of 1403 samples collected from the patients, only 1227 were clinically suspected cases and 176 samples were collected from the consecutive feverish patients. Among the suspected cases only 338 samples were confirmed positive and 889 samples were negative for *Plasmodium species* by PCR. All the 889 samples showed negative result for *plasmodium species* by microscopy and malarial *pf/pv* Ag rapid kits, but only 867 samples were confirmed negative for malarial *pf/pv* Ab rapid kits. Of the 338 PCR positive samples, 337 samples were confirmed positive by microscopy and malarial *pf/pv* Ag rapid kits, but only 284 samples were confirmed positive using malarial *pf/pv* Ab rapid kits. The mean standard deviation for all 1227 samples was 204 ± 71.96, the mean SD of the total 889 clinically proved malaria negative samples were 148.1 ± 50.26 and the mean SD of 338 clinically suspected malaria positive cases was 56.33 ± 22.85. The Confidential interval (95% CI) value for all these clinically (1227 samples) suspected samples was 128.98–280.02, for malaria positive samples the CI value is 32.35–80.32 (338 samples) and for negative samples the CI value was 95.41–200.92 (889). The Standard Error of Mean (SEM) value for all these suspected samples was 29.38 (1227 samples) and for clinically suspected positive samples the SEM value was 9.33 (338 samples) and for negative samples the SEM value was 20.52 (889 samples), respectively. The *P*-value for suspected positive samples is 0.0007 (338 samples) and the *P*-value for negative cases is 0.1470 (889 samples) as shown in the Table 1.

Age distribution

Among all 1227 clinically suspected cases of malaria 197 (16.05%), 285 (23.2%), 255 (20.78%), 226 (18.41%), 186 (115.15%) and 78 (6.35%) patient samples were collected of ages 10 to 20 years, 21–30 years, 31–40 years, 41–50 years, 51–60 years and above 61 years, respectively, as shown in Table 1.

The socio economic status of all the collected patients is described in a graphical representation and all the collected patient samples were distributed gender wise, Area wise, Occupation wise, based on the family size of the collected individuals, based on the education qualification, living status wise, based on the source of the water and based on

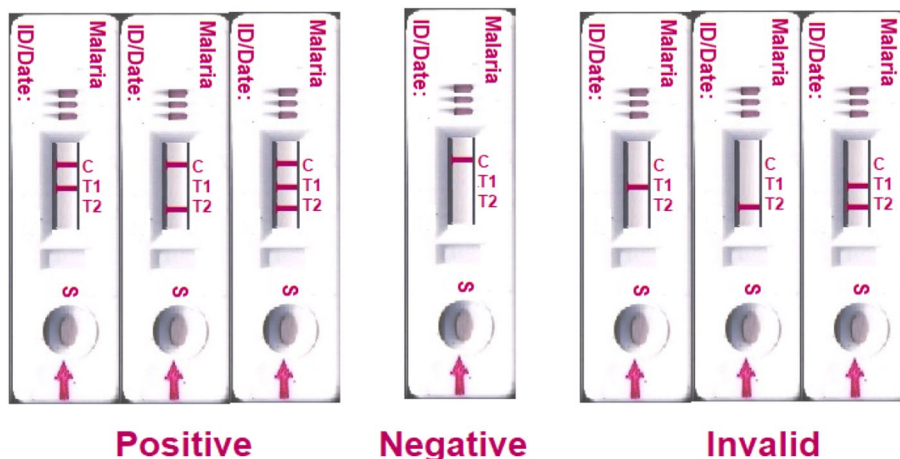**Fig. 2** Interpretation of assay results of lateral flow test kits

Positive   **Negative**   **Invalid**

Table 1 Age-wise distribution among clinically suspected malarial positive and negative cases

Age (in years)	Clinically suspected cases (%) (95% CI)	Malaria positive (%) (95% CI)	Malaria negative (%) (95% CI)
≤ 20	197 (16.05%)	44 (13.01%)	153 (17.21%)
21–30	285 (23.2%)	88 (26.03%)	197 (22.15%)
31–40	255 (20.78%)	72 (21.30%)	183 (20.58%)
41–50	226 (18.41%)	60 (17.75%)	166 (18.67%)
51–60	186 (15.15%)	52 (15.38%)	134 (15.07%)
> 60	78 (6.35%)	22 (6.50%)	56 (6.29%)
Total	1227	338	889
Mean ± SD	204 ± 71.96	56.33 ± 22.85	148.1 ± 50.26
CI (95% CI)	128.98–280.02	32.35–80.32	95.41–200.92
SEM	29.38	9.33	20.52
P-value		0.0007	0.1470

This table explains the age-wise distribution of clinically suspected cases with mean of standard deviation, confidential interval (95% CI), standard error of mean along with the P-value. The P value is calculated in terms of using the total samples statistically significant with malaria positive and negative cases

the preventive and control measures from mosquito bite as shown in the Fig. 3.

Gender distribution

In the present work of the total 1227 suspected cases of malaria, 640 (52%) are males and 587 (48%) are females (Fig. 3a). The clinical diagnostic data for all the suspected cases of malaria were procured from MNR medical college and Hospital, D. All the male and female patients who were clinically suspected of malaria had the symptoms such as fever, chillness, splenomegaly, pallor and convulsions.

Area distribution

Among the 1227 suspected cases of malaria that are distributed and calculated in terms of socio economic status of the malaria disease 732 (60%) patients are from the rural areas and 495 (40%) patients belong to urban area (Fig. 3b).

Occupational distribution

All these clinically suspected cases were distributed based on the type of occupation. 386 patients were daily labour, 327 patients were business people, 106 patients were employees, 273 patients were farmers and the people without work were 135 (Fig. 3c).

Family size distribution

The distribution in terms of family size is, 117 (10%) of patients were < 3 of family size, 365 (30%) patients were with the family size of 3–4 people, 497 (40%) patients with the family size of 4–5 people and the family size is ≥ 6 the number of specimens collected was 248 (20%) (Fig. 3d).

Effect of literacy on disease distribution

The effect of education whether the people are literate or illiterate. 432 (35%) patients were illiterate and about 795 (65%) patients were literate (Fig. 3e).

Effect of living status on disease distribution

The type of living conditions of the individuals was measured based on whether the people were staying in council houses or individual houses or huts or in apartments (Fig. 3f). 193 (16%) were habitats of council houses, 357 (29%) were living in individual houses, 156 (13%) were living in huts and 521 (42%) were living in apartments.

Source of water on disease distribution

Based on the source of water consumption for household purposes, 440 (36%) patients' source of water is from inside the house premises, whereas for around 787 (64%) patients, source of water is from outside the house premises as described in the Fig. 3g.

Effect of prevention and control measures on disease distribution

Based on the prevention of the disease and its control measures from mosquito bite, all the 1227 cases were distributed on the type of the preventive methods for controlling the disease (Fig. 3h).

Evaluation results of diagnostic assays

All the suspected cases were diagnosed using four different methods such as microscopy, PCR assay, Ag lateral flow assay and Ab lateral flow assay. Among all the 1227 clinically suspected cases of malaria, 135 cases were positive for *P. falciparum*, 97 cases were positive for *P. vivax* and 106 number of cases were with mixed infection, i.e. positive for both *P. falciparum* and *P. vivax* and 889 samples were clearly true negatives for malarial parasites. The evaluation of these diagnostic assays reveals the false positive and false negative results affects the sensitivity and specificity and also the treatment. The microscopy and malaria Ag lateral flow assay showed one false
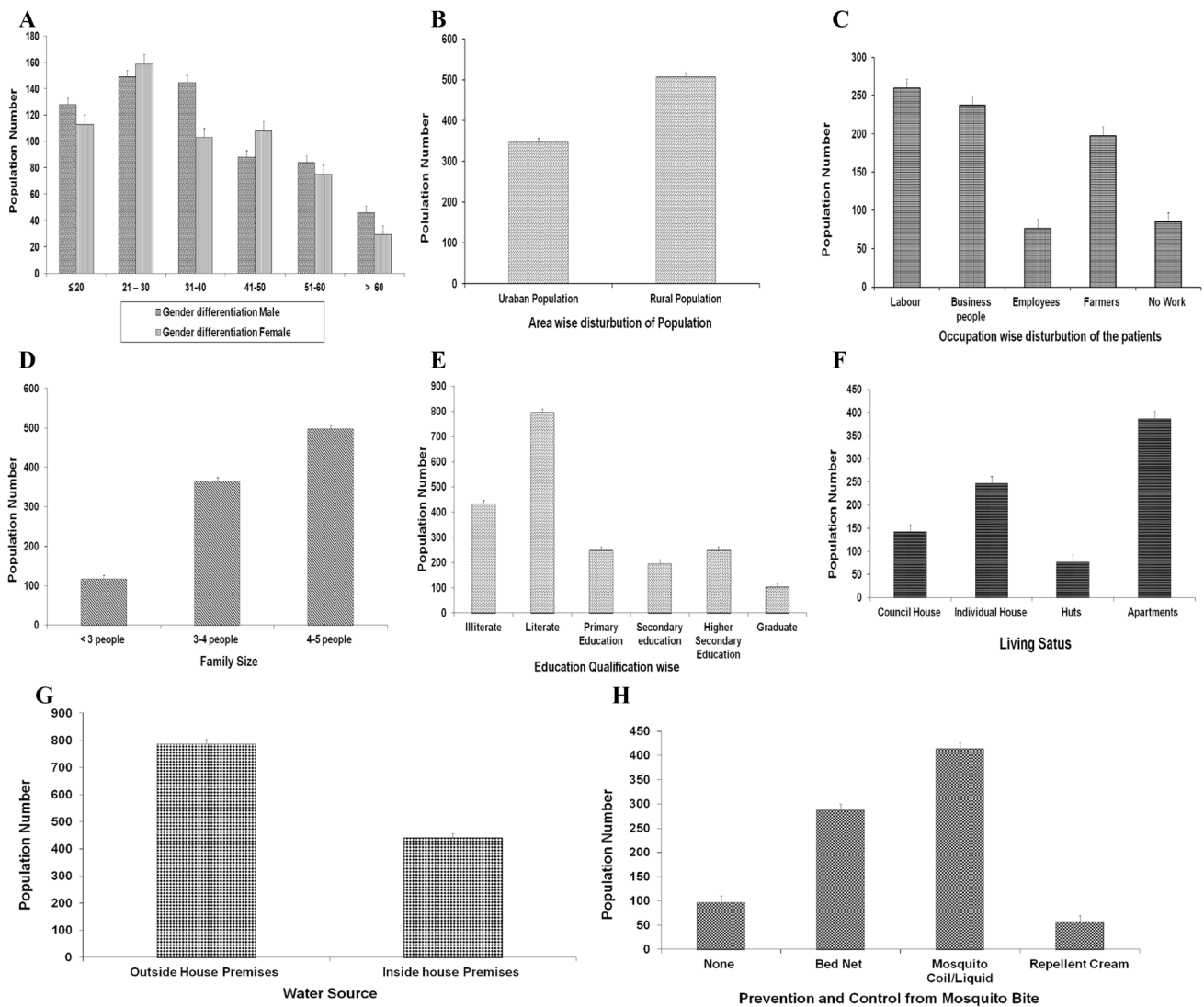
Fig. 3 socio economic status of clinically suspected cases of malaria, **a** gender wise distribution of clinically suspected cases of malaria under socio economic status, **b** area distributions, **c** occupation wise distributions of clinically suspected cases of malaria, **d** family size based distributions of clinically suspected cases of malaria, **e** the effect of education on malaria disease distribution, **f** effect of living status on the distribution of disease, **g** water source wise distributions of disease, **h** prevention and control from mosquito bite

Table 2 Evaluation of the test results of diagnostic assays used for malarial diagnosis

S. no	Name of the detection	Test type	Specimen volume required (µl)	Specimen type serum/whole blood	Total no. of samples ($n = 1227$)		TP	TN	FP	FN
					Total positive	Total negative				
1	Microscopy	Microscopy	100	Whole blood	TP-338	TN-889	337	889	–	01
2	PCR based	Molecular assay	1	DNA	TP-338	TN-889	338	889	–	–
3	Ag based	Lateral flow	5	Wb/plasma	TP-338	TN-889	337	889	–	01
4	Ab based	Lateral flow	5	Wb/plasma	TP-338	TN-889	284	867	54	22

TP true positive (reactive), *TN* true negative (non-reactive), *WB* whole blood, *FP* false positive, *FN* false negative, *Wb* whole blood, *Ag-based* malaria *Pf/Pv* antigen-based detection assay, *Ab-Based* malaria *Pf/Pv* antibody-based detection assay

negative result, whereas PCR assay showed 100% negative and 100% positive results. But malaria Ab rapid kits recorded 54 false positive and 22 false negative results (Table 2).

Statistical analysis

The statistical analysis of the evaluated test kit results were tabulated in terms of the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency. Overall the microscopy and the malaria antigen-based lateral flow assay were more or less similar in accuracy, whereas the PCR assay had 100% sensitivity, specificity, PPV, NPV and efficiency, respectively. But the evolutionary data for malaria antibody lateral flow assay were differing with the standards as shown in the Table 3.

Detection of consecutive feverish patients

Consecutive feverish patients other than malaria were collected from MNR Medical College and Hospital, Sangareddy. 176 samples were collected with different age groups, of which 10 samples showed positive with microscopy, PCR assay and malaria *Pf/Pv* Ag test kits and the remaining 166 samples were negative for malarial parasites. But 36 of 176 samples were positive with malaria *Pf/Pv* Ab kits. Of the positive 36 samples, 10 samples confirmed positive with Microscopy, PCR and *Pf/Pv* Ag kits but the remaining 26 samples were reported false positive with Malaria *Pf/Pv* Ab kits. The fever of the patients may be due to other diseases. All these clinical information was clearly tabulated as shown in the Table 4.

Discussion

The re-emergence of parasitic malaria has renewed interest not only in the preventive measures of the disease, but also in developing a better POC, rapid diagnostic test kit for the diagnosis (Mallepaddi et al. 2016). At present, a number of detecting and diagnosing methods have replaced the conventional microscopy. The impact of malaria is increasing day by day, especially in the tropical endemic areas and so there is a huge demand for rapid diagnostics. Hence, WHO announced the need for developing a rapid, simple, easy-to-use, highly sensitive and accurate, inexpensive POC diagnostic test to determine the existence of parasitic disease in human healthcare (Tangpukdee et al. 2009). According to the scientific research and expert reviews, rapid kit can only meet the present need for diagnosis in the market. The use of rapid kit is very simple, easy to perform, reliable and helps in improving the diagnosis rate in rural endemic areas (Murray et al. 2003; Bell et al. 2005). The diagnosis with rapid test kits in non-endemic regions is becoming more feasible, which may reduce time-to-treatment for cases of imported malaria (Erdman and Cain 2008). Currently, 100 types of malaria rapid diagnostic test kits are available from different manufacturers all over the world (http://www.wpro.who.int/sites/rdt). Most of these kits are antibody based, are specific to *P. falciparum and* hence cannot distinguish the differences between the parasitic species of the malaria individually. The sensitivity and specificity of antigen-based rapid test for diagnosis of malaria has been reported to be an excellent kit compared to the other type of tests (Durand et al. 2005; Kyabayinze et al. 2010; Mallepaddi et al. 2016).

The present study explains the drawbacks of improper diagnostic kits causing misdiagnosis for malaria (Bell

Table 3 Comparison of parameters between diagnostic assays

S. no.	Name of the method	Sensitivity %	Specificity %	PPV (%)	NPV (%)	Efficiency (%)
1	Microscopy	99.70	100	100	99.88	99.91
2	PCR assay	100	100	100	100	100
2	Ag based	99.70	100	100	99.88	99.91
3	Ab based	92.81	94.13	84.02	97.52	93.80

Table 4 The Results of Consecutive feverish patients other than malaria

S. no.	Name of the detection	Total no. of feverish samples (*n* = 176)		TP	TN	FP	FN
		Total positive	Total negative				
1	Microscopy	TP-10	TN-166	10	166	–	–
2	PCR method	TP-10	TN-166	10	166	–	–
3	Ag-based LFT	TP-10	TN-166	10	166	–	–
4	Ab-based LFT	TP-10	TN-166	10	140	26	–

et al. 2005; Murray et al. 2009). Mainly the disease diagnosis should focus on which diagnostic method will give proper and accurate diagnosis results. Because of the presence of the species-specific antigenic protein in blood of the affected people the use of antigen-based kits will deliver proper diagnosis. The results of statistical analysis of the evaluated test kit results are tabulated in terms of sensitivity, specificity, PPV (positive predictive value), NPV (negative predictive value) and efficiency. Overall the microscopy and the malaria antigen-based lateral flow assay exhibit similar sensitivity, i.e. both the assays had 99.70% sensitivity, 100% specificity, 100% PPV, 99.88% NPV and 99.91% efficiency, respectively, whereas the PCR assay had 100% sensitivity, 100% specificity, 100% PPV, 100% NPV and 100% efficiency. But the evolutionary data for malaria antibody lateral flow assay have 92.81% sensitivity, 94.13% specificity, 84.02% PPV, 97.52% NPV and 93.80% efficiency as shown in the Table 3.

The results obtained in the present study were in agreement with the study carried out by Murray et al. (2003) and Bell et al. (2005) in terms of variation in the sensitivity and specificity with antibody kits (Kyabayinze et al. 2012). In our study the antibody kits were compared with gold standard microscopy, PCR assay and with antigen rapid kits. The antibody test kits did not show promising results and variation in the sensitivity and specificity and false positive and false negative results was reported. The major problem with antibody-based kits was the cross reactivity. The study of cross reactivity with malaria antibody rapid kits was explained with rheumatoid factor in blood that generates a false positive test line at early stages of the diagnosis (Laferl et al. 1997; Grobusch et al. 1999). The cross-reactivity that occurs is may be due to heterophile antibodies (Wellems et al. 1991; Moody 2002; Biswas et al. 2005; Laferl et al. 1997). In addition, Grobusch et al. (1999) and Chansuda et al. (2008) reported that false positive results could occur due to cross-reactivity with a limited number of antibody kits of malaria. According to Navin kumar et al. (2012) and Bharathi et al. (2016) study reported due to the genetic variation in Histidine rich proteins pfhrp2 and/or pfhrp3 gene deletion among Indian Plasmodium falciparum population there is a possible cause of variable sensitivity of malaria rapid diagnostic tests. But in this present study the malarial *pf/pv* Ag rapid diagnostic test kit showed promising results with high levels of specificity and sensitivity that were compared to that of microscopy and PCR assay which were similar to the results reported by Beadle et al. (1994), Bharti et al. (2008), Mallepaddi et al. (2016). Therefore, the present study proved that malaria *pf/pv* antigen rapid detection test kit is an effective and sensitive tool and is the better diagnostic test for detecting the malarial parasites, especially in resource-limited point of care areas.

Conclusion

The present work reveals that rapid diagnostic kits are very simple, inexpensive, user-friendly, point of care and effective diagnostic assays that can be executed at the bedside for detection of malaria. The sensitivity, specificity, PPV, NPV and efficiency of Ag-based malaria rapid kits were similar to that of conventional microscopy and do not require highly skilled personnel to perform or interpret the results. Early diagnosis and treatment are imperative in preventing the complications. Microscopy is the gold standard for malaria parasite detection but is laborious and requires experts to interpret the results. Rapid immunochromatographic antigen-based test that detects specific antigens produced by malaria parasite in the blood can be performed at bedside in 10–15 min.

Compliance with ethical standards

Ethical clearance The study was approved by the Organizational Human Ethical Committee.

Conflict of interest There is no conflict of interest related to this study.

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