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Original Research Article

# Rapid Diagnostic Tests in Comparison with Multiplex Polymerase Chain Reaction for Early Diagnosis of Malaria, Dengue and Chikungunya in Cases of Acute Febrile Illness

**Kilikdar Mousumi[*1], Jampala Srinivas[2], Ansari Aman[3], Siraj Safiya[4], Chitrans Pallavi[5], Rehman Zainab[6]**

[1]Associate Professor, Department of Microbiology, Rajshree Medical Research Institute, Bareilly (UP), India

[2]Professor, Department of Microbiology, Rajshree Medical Research Institute, Bareilly (UP), India

[3,4]Junior Resident, , Department of Microbiology, Rajshree Medical Research Institute, Bareilly (UP), India

[5]Tutor, Department of Microbiology, Rajshree Medical Research Institute, Bareilly (UP), India

[6] Senior Resident, Department of Microbiology, Rajshree Medical Research Institute, Bareilly (UP), India

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**Corresponding author: Dr. Mousumi Kilikdar**

**Conflict of interest: Nil**

**Abstract:**

**Introduction:** Malaria, dengue (DENV) and chikungunya (CHIKV) are one of the most common causes of acute febrile illness (AFI). As they share some common clinical features, it becomes imperative to confirm the diagnosis of these febrile illnesses at the earliest by performing appropriate laboratory tests. This will establish a specific diagnosis with specific treatment.

**Aim:** This study was conducted to evaluate the diagnostic value of various rapid diagnostic tests (RDTs) in comparison with Multiplex PCR for the early diagnosis of Malaria, Dengue and Chikungunya in cases of AFI.

**Materials and Methods:** A cross sectional and analytical study was conducted to evaluate various RDTs for early diagnosis of DENV, malaria and CHIKV in AFI cases admitted to a tertiary care hospital over a period of 12 months. Blood samples were tested by RDTs and multiplex PCR for diagnosis of DENV, malaria and CHIKV.

**Results:** Out of total 200 AFI cases, 63 were positive for DENV, malaria and CHIKV by RDT (31%) out of which 38 patients were also positive by PCR (19%) and 25 patients (12.5%) were only positive by RDT but negative by PCR while comparing RDT and PCR. The diagnostic value of RDT for DENV, malaria and CHIKV were evaluated using PCR as the reference standard. The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of DENV RDT was 76.3 %, 100 %, 100 % and 94.7% respectively. Whereas the sensitivity, specificity, PPV and NPV of malaria RDT was found to be 34.8%, 100%, 100% and 92.2% respectively. We observed that the sensitivity, specificity, PPV and NPV of CHIKV RDT was 50%, 100%, 100% and 99.5% respectively.

**Conclusion:** Early and accurate diagnosis plays a crucial role in management of AFI cases and thus preventing morbidity and mortality. In this study, various RDTs were evaluated using PCR as the reference standard. Sensitivity of RDT was found to be low. Though RDTs and PCR are useful for early diagnosis, RDT are better in terms of rapidity, cost and

simplicity. Hence, RDTs should be used in conjunction with reference standard for better prognosis of AFI cases.

**Keywords:** RDT, multiplex PCR, AFI



## Introduction

AFI is defined as illness of less than one week duration with no identified source [1,2] . It is a presenting feature of malaria, dengue, chikungunya, leptospirosis, typhoid, Japanese encephalitis, rickettsial infections and Influenza. [3]

It is imperative to confirm diagnoses of febrile illnesses by performing appropriate laboratory investigations, in order to establish a specific diagnosis and give proper treatment accordingly [4]. Malaria, Dengue and Chikungunya have some common clinical features, such as, pyrexia, arthralgia, vomiting, headache & fatigue, but differ in their clinical outcomes [5]. There are very high chances of misdiagnosis of these febrile illnesses [6,7,8,9].

As per World Malaria Report 2021, globally, deaths due to malaria in 2020, expanded to an estimated 62700, a 12% increase from 2019 [10]. In 2020, 83% of all malaria cases in WHO South-East Asia Region were accounted for by India [10]. Certain arboviruses like DENV and CHIKV cause vast epidemic outbreaks all over the world [11]. As per WHO statistics (2022), deaths due to dengue raised from 960 to 4032.[11] During 2020 and 2021, total number of cases and reported deaths due to dengue apparently dropped down [11]. In many countries, COVID-19 pandemic might have hindered reporting of cases. In 2017, a total of 548 cases of Chikungunya, were reported by European Centre for disease prevention and control of which 84% were confirmed cases [12]. Outbreaks of Chikungunya were also reported in Sudan (2018), Yemen (2019) and Cambodia and Chad (2020) [12].

In a recent systematic review, dengue, malaria & chikungunya co-infection cases were seen; it also revealed that the most common co-infection was dengue and malaria co-infection, followed by the chikungunya/dengue chikungunya/malaria and dengue/malaria/chikungunya co-infections [13]. Co-infection among AFIs are prevalent and may have serious implications, as seen in some studies [14, 15].

With the advent of various diagnostic tools that are available for the diagnosis of malaria, DENV and CHIKV, RDTs are easy to use point of care diagnostic tests with limitation like varying sensitivity and specificity. Whereas a highly valuable and distinguishing test between various pathogens causing AFI is multiplex PCR assays which can detect multiple pathogen in a single sample [16]. Also PCR assays are reported to have good specificity and varying sensitivity [16]. With this context, the study was conducted to assess the diagnostic value of RDTs in comparison with Multiplex PCR for the early diagnosis of Malaria, Dengue and Chikungunya.

## Materials & Methods:

**Place of the study:** Department of Microbiology, Rajshree Medical Research Institute and Hospital, Bareilly.

**Study design:** Cross-sectional, analytical study.

**Study period:** 12 months from October 01, 2021 to September 30, 2022.

**Sample size:**

$N=Z^2 P (1-P)/ E^2$
Z=Standard normal variate
P=Prevalence rate, E=Absolute error,

N=required sample
Prevalence of malaria and dengue in cases of acute undifferentiated fever in 6 states of India (2017) was 16% from Mørch et al [17].
Here, Z=1.96 at 95% confidence interval, P=16%, E=5%
N= (1.96 x 1.96) x 0.16 x (1-0.16)/ (0.05x 0.05)
N = 206 with 95% confidence level and 5% absolute precision.
Therefore calculated sample size is 200 patients.

**Study population**

**Inclusion criteria:** All patients with acute febrile illness.

**Exclusion criteria:**

1. Patients who have already taken antimalarial treatment during the febrile period.
2. All patients positive for leptospirosis and typhoid using standard diagnostic tests were excluded from the study.

3 ml of blood each was collected in both sterile EDTA tube and plain tube from a single patient. Blood sample was centrifuged to separate plasma and serum which was used for PCR and RDT respectively. However for performing rapid test of Malaria, whole blood sample from EDTA tube was used. Separated plasma was stored at -80 $^0$ C until further testing. Demographic details of patients were collected from hospital records system. Institutional Ethical committee permission was obtained to conduct the study.

**Multiplex PCR for detection of Malaria, Dengue and Chikungunya**

Molecular diagnoses of the cases of acute febrile illnesses were performed by Real time PCR at the molecular laboratory of Microbiology Department which is a Bio-Safety Level- II laboratory.

RNA/DNA was extracted from plasma manually by TRU PCR total nucleic acid extraction kit according to manufacturer's instructions. The amplification and detection of pathogen RNA/DNA were performed on Quant Studio 5 molecular system (Applied Biosystems, Waltham, Massachusetts) using TRU PCR Dengue, Chikungunya, Malaria detection kit.

The amplification/detection is based on the principle of Real Time PCR, which simultaneously amplifies and detects DENV RNA, CHIKV RNA and Plasmodium spp. DNA. The fluorescent channels FAM, HEX and VIC were used to detect DENV, CHIKV, Plasmodium spp. respectively whereas ROX channel was used to detect the fluorescent signal of endogenous internal control (IC). Positive and negative controls were used in each run.

The PCR assay was performed using the following thermocycler protocol: Reverse transcription at 50 $^0$ C for 20 minutes, initial denaturation at 94 $^0$ C for 10 minute, denaturation at 94 $^0$ C for 15 seconds, annealing, extension and fluorescent signal collection at 59 $^0$ C for 45 seconds.

**Rapid diagnostic test**

**1-ErbaQik Malaria pf/pan Ag test (Transasia Bio-Medicals LTD, Daman, India)**
It is a solid phase immuno-chromatographic assay for the rapid, qualitative detection and differentiation of P. falciparum histidine rich proteinII (HRPII) and Plasmodium spp. Lactate dehydrogenase (pLDH) (P.vivax, P. malariae and P. ovale) from whole blood specimen.

**2-Dengue Day 1 test (J. Mitra LTD, Delhi, India)**
It is an immuno-chromatographic test for the rapid and qualitative detection of Dengue NS1 antigen and differential detection of IgM and IgG antibodies to dengue virus in human serum/plasma.

**3- Advantage Chikungunya IgM Card test (J. Mitra LTD, Delhi, India)**

It is a rapid, immuno-chromatographic assay that detects Chikungunya specific IgM antibody in human serum/plasma.

**Statistical analysis**

The diagnostic test for DENV, Malaria, and CHIKV (PCR and RDT) were assessed for sensitivities, specificities, PPV and NPV, likelihood ratios and odds ratio [with 95% confidence intervals]. Significance was assigned at $p<0.05$ for all parameters. All statistical analysis was performed using SPSS (IBM SPSS Statistics version 29).

**Results:**

In our study, samples were collected from 200 participants with acute febrile illnesses of which 137/200 (68.5%) were males and 63/200 (31.5%) were females (Table 1). The mean age was 38.54 years (Table 1). The hematological and biochemical parameters (mean) were within normal limits.

Out of total 200 AFI cases, 63 were positive for DENV, malaria and CHIKV by RDT (31%) out of which 38 patients were also positive by PCR (19%) and 25 patients (12.5%) were only positive by RDT but negative by PCR while comparing RDT and PCR. Among the RDT positive cases we observed a higher level of total leucocyte count (TLC) (19/63, 30%), leucopenia (4/63, 6.3%). Similarly lower platelet counts were noted among 23 (36.5%) patients.

Median duration of fever among all participants was 3 days (Table 1). Majority of the diagnostic test positivity was observed to be in early stage of the illness (Table 2).

Positive detection rate was higher with RDT (63/200, 31%) as compared to PCR (38/200, 19%). The diagnostic value of RDT for DENV, malaria and CHIKV were evaluated using PCR as the reference standard. The sensitivity and specificity of DENV RDT was 76.3 % (95 % CI, 59.8% - 88.6%) and 100 % (95 % CI, 97.7% - 100%) respectively (Fig 1) with corresponding positive and negative predictive values of 100 % (95 % CI, 88.1% - 100%) and 94.7% (95% CI, 90.2% - 97.6%) (Table 3). Overall agreement with reference test was 95.5% with significant P value.

Whereas the sensitivity and specificity of malaria RDT was 34.8% (95% CI, 16.4% - 57.3%) and 100% (95% CI, 97.9% - 100%) respectively (Fig 1) in association with positive and negative predictive values of 100% (63.1% - 100%) and 92.2% (87.4% - 95.6%) respectively (Table 4). 92.5% agreement was found with PCR with significant P value. In this study, we observed that the sensitivity, specificity, PPV and NPV of CHIKV RDT was 50% (1.26% -98.7%), 100% (98.2% - 100%) (Fig 1), 100% (2.5% - 100%) and 99.5% (97.2% - 100%) respectively (Table 5). 99.5% agreement was found with PCR with significant P value.

**Table 1: Characteristics of the study Participants (N=200)**

| Characteristics | Mean (SD) or Median (IQR) |
|---|---|
| Mean Age (SD) | 38.54(12.45) |
| Male (%) | 137(68.5) |
| Mean WBC (SD) in Cells/cumm | 8688(2954) |
| Mean Platelet count (SD) in lacs/cumm | 1.77(0.70) |
| Median Total Bilirubin (IQR) in mg/dl | 0.6(0.5-0.8) |
| Median Direct bilirubin (IQR) in mg/dl | 0.23(0.14-0.34) |
| Mean Total Protein (SD) in gm/dl | 6.19(1.17) |
| Mean Albumin (SD) in gm/dl | 3.81(1.06) |
| Median AST (IQR) in U/L | 57(54-86.2) |

| | |
|---|---|
| Median ALT(IQR) in U/L | 49.3(45.3-66.3) |
| Mean Alkaline Phosphatase (SD) in IU/L | 110.9(37.15) |
| Median Serum Creatinine (IQR) in mg/dl | 0.9(0.7-1.2) |
| Median duration of Fever in days(IQR) | 3(2-4) |

**Table 2: Positivity ofRDTandmultiplexPCRinearly(1to7days)andlate(8to14days)stagesofacutefebrileillness**

| Day post onset of illness (DPO) | RDT Dengue | | RDT Malaria | RDT CHIK | Multiplex PCR |
|---|---|---|---|---|---|
| | NS1 | IgM | | | |
| 1-7days | 30/38(78.9%) | - | 21/23(91%) | 2/2(100%) | 32/38(84.2%) |
| 8-14days | - | 8/38(21%) | 2/23(8.6%) | - | 6/38(15.7%) |

**Table 3: Diagnostic accuracy of RDT for DENV**

| | RDT | | |
|---|---|---|---|
| **PCR** | **Negative** | **Positive** | **Total** |
| Negative | 162 | 9 | 171 |
| Positive | 0 | 29 | 29 |
| Total | 162 | 38 | 200 |

| Sensitivity (95%CI) | Specificity (95%CI) | PPV (95%CI) | NPV (95%CI) | Likelihood ratio(+) | Likelihood ratio(-) | Accuracy |
|---|---|---|---|---|---|---|
| 76.3%(59.8%-88.6%) | 100%(97.7%-100%) | 100%(88.1%-100%) | 94.7%(90.2%-97.6%) | - | 0.24(0.13-0.42) | 95.5%(92.6%-98.4%) |

| | |
|---|---|
| Observed Agreement | 95.50% |
| Expected Agreement | 72.01% |
| Kappa | 0.8392 |
| Std.Err. | 0.0698 |
| Z | 12.02 |
| P-value | <0.001 |

**Table 4: Diagnostic accuracy of RDT for Malaria**

| | RDT | | |
|---|---|---|---|
| **PCR** | **Negative** | **Positive** | **Total** |
| Negative | 177 | 15 | 192 |
| Positive | 0 | 8 | 29 |
| Total | 177 | 23 | 200 |

| Sensitivity(95%CI) | Specificity(95%CI) | PPV (95%CI) | NPV(95%CI) | Likelihoodratio(+) | Likelihood ratio(-) | Accuracy |
|---|---|---|---|---|---|---|
| 34.8%(16.4%-57.3%) | 100%(97.9%-100%) | 100%(63.1%-100%) | 92.2%(87.4%-95.6%) | - | 0.65(0.48-0.88) | 92.5%(88.8%-96.2%) |

| | |
|---|---|
| Observed Agreement | 92.50% |
| Expected Agreement | 85.42% |
| Kappa | 0.4856 |
| Std.Err. | 0.0606 |

| | |
|---|---|
| Z | 8.01 |
| P-value | <0.001 |

**Table 5: Diagnostic accuracy of RDT for Chikungunya**

| | RDT | | |
|---|---|---|---|
| **PCR** | **Negative** | **Positive** | **Total** |
| Negative | 198 | 1 | 199 |
| Positive | 0 | 1 | 1 |
| Total | 198 | 2 | 200 |

| **Sensitivity (95%CI)** | **Specificity (95%CI)** | **PPV (95%CI)** | **NPV (95%CI)** | **Likelihood ratio(+)** | **Likelihood ratio(-)** | **Accuracy** |
|---|---|---|---|---|---|---|
| 50%(1.26%-98.7%) | 100%(98.2%-100%) | 100%(2.5%-100%) | 99.5%(97.2%-100%) | - | 0.65(0.48-0.88) | 99.5%(98.5%-100%) |

| | |
|---|---|
| Observed Agreement | 99.50% |
| Expected Agreement | 98.51% |
| Kappa | 0.6644 |
| Std.Err. | 0.0666 |
| Z | 9.97 |
| P-value | <0.001 |

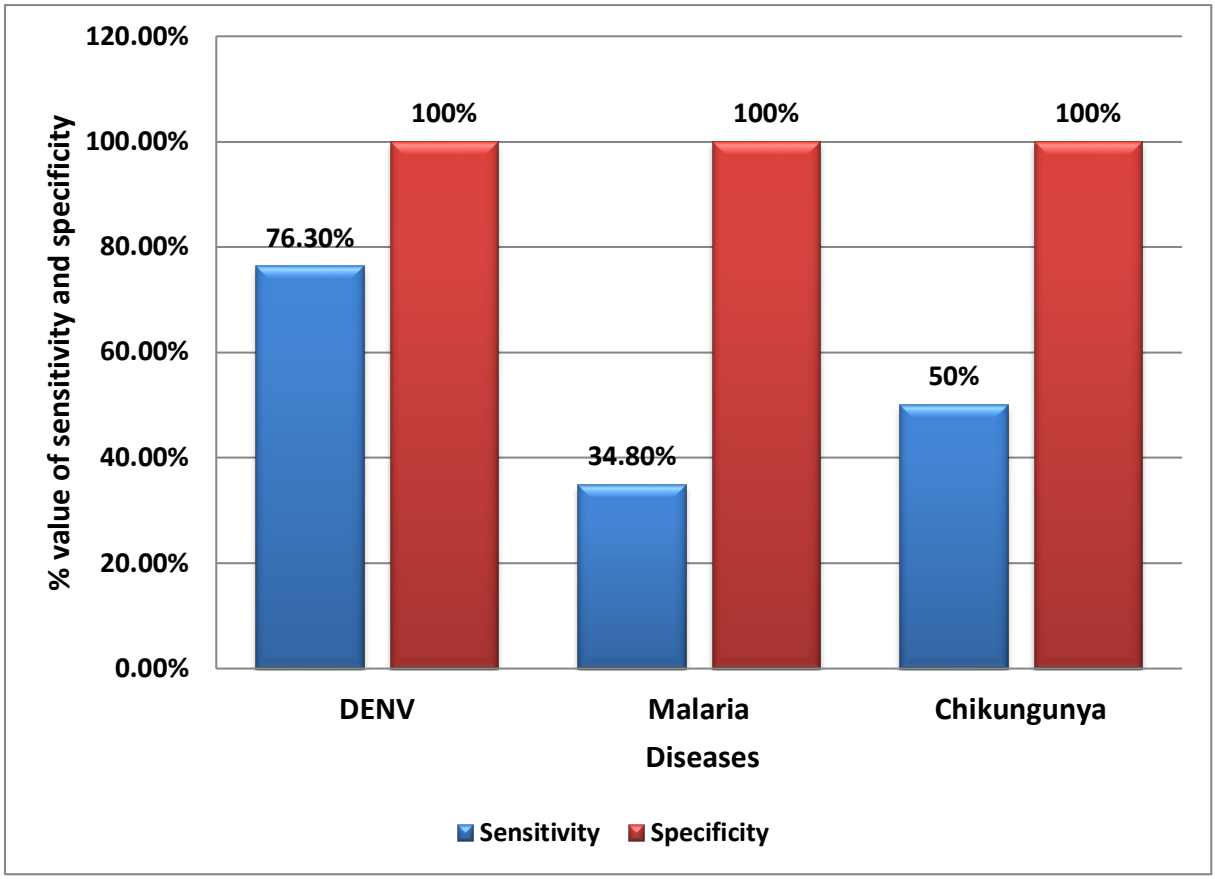


**Figure1:SensitivityandspecificityofRDTforDENV,Malariaand Chikungunya**

## Discussion

In this study period of 12 months the most common etiology of AFI was found to be dengue. The seasonality of dengue followed a pattern with a period of high transmission that begins as early as June and lasts until the end of November. This is correlating with the studies like Lorenzi O et al [18] and Robinson ML et al [19] who also reported dengue as the most common cause of AFI with seasonality.

Male preponderance was noted in our study which is attributed to occupational and recreational exposure of males to mosquitoes. Leucocytosis was observed in our study which is in contrast with study of Budihal N [20] who reported leucopenia in most of AFI cases. We noted thrombocytopenia in most of the cases as also observed by of Budihal N.[20]

## Dengue

In the current study the prevalence of dengue was found to be the highest (14.5 %) which is in line with other studies like Siddique O et al [21], Vikram K et al [22] and Morch et al.[17] Dengue virus produces non-structural protein 1 (NS1) during the viremic early phase of infection and after more than five days IgM can be detected. Combination RDTs employ the detection of both NS1 antigen and IgM/IgG routinely in diagnostics, as they have high sensitivity in both phases.[23] Although IgM antibodies are more prone to give cross reactivity than NS1 antigen, combination tests have shown some false positive reactions in AFI cases especially in chikungunya.[17]

In our study, the sensitivity of dengue RDT was found to be 76.3 % which is in line with Tricou V et al [24] but not correlating with Kok Siang et al [25] who reported sensitivity of 92.6%. Specificity, positive and negative predictive values reported 100 %, 100 % and 94.7% respectively which is correlating with Kok Siang et al.[25] In our study, we observed false positivity of RDT in 9 samples considering PCR as reference test. False positivity could be the result of cross reactivity with other flavivirus and arthropod borne infections such as Yellow fever, Japanese encephalitis, West nile virus etc [24]. Hence, test results must be correlated clinically otherwise over diagnosis could impart serious implications on public health. Nevertheless, RDTs can be useful in early and quick confirmation of dengue infections ultimately strengthening disease surveillance program.

## Malaria

In our study, prevalence of malaria infection was found to be 4% (8/200). Microscopy being the gold standard test in malaria diagnosis, still suffers from few disadvantages like low parasitemia, high expertise and it is time consuming. Hence it is better to go along with RDTs or PCR.[26] We reported sensitivity of malaria RDT to be 34.8% which is close to the sensitivity of 49% reported by Shankar et al [27] and specificity, positive and negative predictive values of 100%, 100% and 92.2% respectively which is in line with Ahmad et al [28].

Malaria parasites were detected by PCR in 4% (8/200) and by RDT 11.5% (23/200) among the AFI cases. All the RDT positive cases were confirmed by PCR except 15 samples which were negative by PCR. We found high false positivity rate with RDT (PCR was used as reference standard) which could be a major concern for public health. False positivity exposes healthy individuals to unnecessary administration of drugs further adding to drug resistance.[27] Hence, deploying RDTs for malaria diagnosis needs caution.

## Chikungunya

In this study, we observed only one case of Chikungunya 0.5% (1/200) which was positive by both RDT, PCR and co infected with dengue. One false positive

case was noted by RDT considering PCR as reference standard.

Poor sensitivity of chikungunya RDT was observed as also seen by Prat CM et al [29]. Specificity, positive and negative predictive values noted in our study was higher than values reported by Prat CM et al [29].

In terms of usefulness in clinical practice, multiplex-PCR can provide the clinicians a prompt and accurate diagnosis of acute febrile illness so that early treatment can be initiated. Moreover, the assay provides confirmation in cases with false positive or false negative RDT results. Another advantage with multiplex PCR is it allows comprehensive testing of multiple pathogens in a shorter time frame whereas in traditional (single plex) PCR a clinician has to order each test independently.

This study evaluated the performance of various RDTs against multiplex PCR in diagnosis of dengue, malaria and chikungunya. High false positivity and varying sensitivity was observed with RDTs. Efforts are being made continuously to improve sensitivity and specificity of various RDTs for accurate and better management of AFI cases. Hence interpretation of RDTs should be done in conjunction with a reference standard and clinical diagnosis.

## Limitations

In the present study, various RDTs were evaluated using PCR as the reference standard. The sensitivity was low as it was based on the assumption that the cases detected by RDTs but not PCR were false positive. This should be kept in mind that the possibility that negative PCR could have been due to the presence of PCR inhibitors as no attempt was made to look for such inhibitors in this study.

## Conclusion

Dengue was found to be the most prevalent infection among AFI cases. Early diagnosis plays a huge role in individual case management and thus preventing morbidity and mortality. Various RDTs were evaluated using PCR as the reference standard. Sensitivity reported was low**.** RDT are better in terms of rapidity, cost and ease, although both are useful for early diagnosis.

Hence, RDTs should be used in conjunction with reference standard for better prognosis of AFI cases.

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