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A PILOT STUDY OF ACUTE UNDIFFERENTIATED FEVER USING CERTAIN RAPID MICROBIOLOGICAL AND VIROLOGICAL TESTS

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ABSTRACT

Acute Undifferentiated Fever is an emerging problem in India especially during the monsoon months. This study was conducted as a prospective observational study in a tertiary care center to determine the etiology of AUFI. Thirty patients with acute onset fever of less than 10 days duration without any localizing signs were included in the study. Dengue was found to be the commonest cause of AUFI in young adults and also in under fives in monsoon months. Leptospirosis was found to be the next common cause of AUFI. Scrub typhus is also increasingly being reported as a cause of AUFI as we have documented in our study. A large number 36.7% (n=11) of our patients reported dual infections. The predominant group 45.5% (n=5) with dual infections were among those with leptospirosis and scrub typhus and the next common dual infection found was among leptospirosis and dengue. Derangement of liver enzymes along with the positivity of one of rapid tests may be considered as a marker for dual infections suggesting need for further evaluation.

KEYWORDS: Acute Undifferentiated Fever, Serological tests, RT-PCR, Dual infections

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INTRODUCTION

Acute undifferentiated fever is a fever without specific symptoms or localizing signs and is the commonest early feature of many infections. Recent reports suggest that the common causes of AUFI in tropical countries are Scrub typhus, Malaria, Enteric fever, Dengue, Leptospirosis, Chikungunya, Spotted fever Rickettsiosis, Hantavirus, Q fever, Brucellosis, Influenza, Venezuelan equine encephalitis (VEE) and other bacterial infections like respiratory, urinary and diarrheal illness [15,5]Persons of all ages and races are susceptible to these AUFI; most often young adult males are more frequently infected by these illnesses than females, with increased numbers of deaths noted among adult men. Burden of the diseases for these common illnesses remains unknown largely because of lack of proper fever diagnostics, also studies on AUFI are poorly funded[4.] In view of atypical clinical manifestations and in the absence of any localizing signs, the diagnosis of AUFI is often clinical with serological diagnosis being the mainstay for definitive diagnosis of these diseases. Missed diagnosis / wrong diagnosis of these AUFI also pose a greater impact in terms of complications[6]. Thus there is a need for early detection of various etiological agents that may cause these types of febrile illnesses. There are various rapid diagnostic tests available for diagnosis of these diseases, however there is little data on the performance of these tests in acute care settings.This study was done to delineate the etiology of acute undifferentiated fever in monsoon season and to review the diagnostic tools that are currently available or in development and the potential role of these rapid Microbiological and Virological tests for Dengue, Malaria, Leptospirosis, Scrub typhus, Chikungunya in case detection & identification of markers of severe disease. This study mainly focuses on Dengue, Malaria, Leptospirosis, Scrub typhus and Chikungunya which lead to a significant level of morbidity among the population, but along with the morbidity they are also an important and major cause of mortality in the patients.

MATERIALS AND METHODS

A prospective study was undertaken in a tertiary care center on patients presenting with acute undifferentiated febrile illness.

Study subjects

Patients presenting with fever of less than 10 days duration were enrolled, during the study period of 3 months. This study included both adult and pediatric patients and all the patients enrolled were in-patients. The patients were selected based on the inclusion criteria given below. The enrolled study subjects presented with all the symptoms of inclusion criteria. Presence of any localizing signs like respiratory, urinary and diarrheal illness was considered as an exclusion criteria. None of these patients had any evidence of samples which were bacteriologically proven to be culture positive.

Inclusion criteria

Fever of < 10 days duration, maculopapular rash, myalgia, headache, nausea, vomiting, joint pains, body pain, petechiae. Proforma was designed and patient's details were collected accordingly. Proforma included patients details like name, age, sex, occupation and also about symptoms like fever, jointpains, bodyache, maculopapular – rash, lymphadenopathy, hepatosplenomegaly, headache, abdominal symptoms**,** hemorrhagic manifestations etc including laboratory details such as the platelet count, haemoglobin, total count, differential count, MP& MF, Liver and renal function tests and radiological investigations such as CT/MRI/USG.

Specimen collection & processing

Patient's serum sample was collected for this study and aliquoted and then frozen at - 20ºc before processing and subjected to *in-vitro* rapid diagnostic tests for all ; Dengue , Scrub typhus , Leptospirosis , Malaria and CHIKV. Molecular method was included for Dengue and CHIKV only.

Methodology

a. Dengue

Dengue virus was tested using Dengue NS1 Ag and Ab Combi Card from Diagnostic Enterprises, Himachal Pradesh, India , lot no : 127047 and by conventional in-house RT-PCR.Qiagen mini viral RNA (Qiagen, USA) kit was used for RNA extraction. A one step RT-PCR was carried out using Qiagen One step RT-PCR (Qiagen, USA) kit on a thermo cycler (Applied Biosystems, Veritticycler)

Forward primer Dl 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'

Reverse primer D2 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'

The target amplicon size is 511bp. RT–PCR amplicons products were run through 2.5 % agarose gels using 100 bp ladder (*Biolabs,* U.S.A as a DNA size marker). Suitable positive and negative controls were used.

b. Chikungunya

A one step RT-PCR was carried out using Qiagen One step RT-PCR (Qiagen, USA) kit on a thermo cycler (Applied Biosystems, Veritticycler).The RNA extracted as outlined previously was used to perform this assay.

The primers used in the study:

CHIK1 – Forward - TATCCTGACCACCCAACGCTCC

CHIK 2 – Reverse - ACATGCACATCCCACCTGCC

The target (E2 region) amplicon size is 305bp. RT–PCR amplicons products were run through 1 % agarose gel using 100 bp ladder (*Biolabs,* U.S.A as a DNA size marker). The amplification product 305 bp lies within the gene that code for viral envelope protein E2. Suitable positive and negative controls were used.

c. Leptospirosis

Leptospirosis was tested using Lepto IgM Microlisa. It is a microwell ELISA test for *in - vitro* qualitative detection of leptospira specific IgM antibody in human serum / plasma manufactured by J.Mitra and Co.Pvt.Ltd, New Delhi, India, lot no: 228006.

d. Scrub typhus

Scrub typhus was tested using SD Bioline Tsutsugamushi Test from S.D Standard Diagnostics, INC, Korea, lot no: 065014. It is a solid phase immunochromatographic assay for rapid, qualitative detection of IgG , IgM , IgA antibodies to *O. tsutsugamushi* in human serum, plasma or whole blood.

e. Malaria

Malaria was tested using OnSite Malaria Pf/ Pv Ab Rapid Test Cassette from CTK Biotech, Inc USA, lot no: No 90050. It is a lateral flow chromatographic immunoassay for the simultaneous detection and differentiation of antibodies including IgG, IgM and IgA to *Plasmodium falciparum* (Pf) and *vivax , ovale* and *malariae* in human serum or plasma.

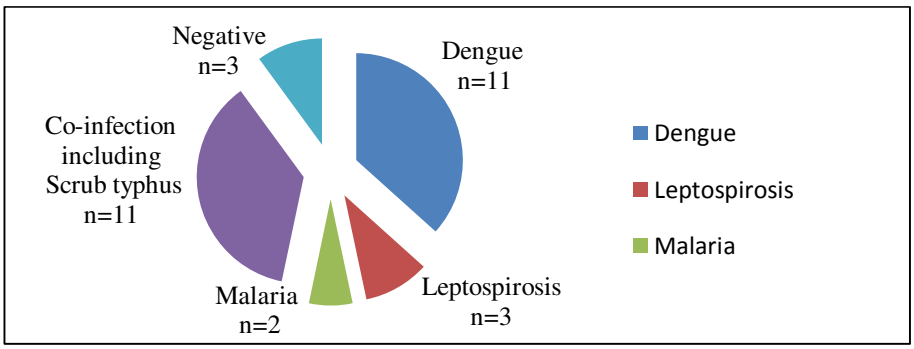
RESULTS

Among all the thirty patients enrolled in the study, 20 adults and 10 paediatric patients were included. The age of the patients ranged from 2 years to 50 years. Majority i.e. 65% (n=13) of the adult patients with acute fever were in the age group of 18 to 40 years. 40% (n=4) of the paediatric patients with acute fever were less than five years of age. The male to female ratio was found to be 1.7: 1.

Etiological profile of acute undifferentiated fever

Patient's sera was analysed using rapid serological tests for Dengue, Leptospirosis, Malaria and Scrub typhus, as well as by molecular methods for dengue and CHIKV. Among the thirty patients enrolled 36.6% (n = 11) were positive for dengue, 10% (n=3) patients tested positive for leptospirosis, and 6.6% (n=2) patients tested positive for Malaria. Only three (10%) of patients enrolled tested negative to all the rapid tests including molecular tests (Fig.1)

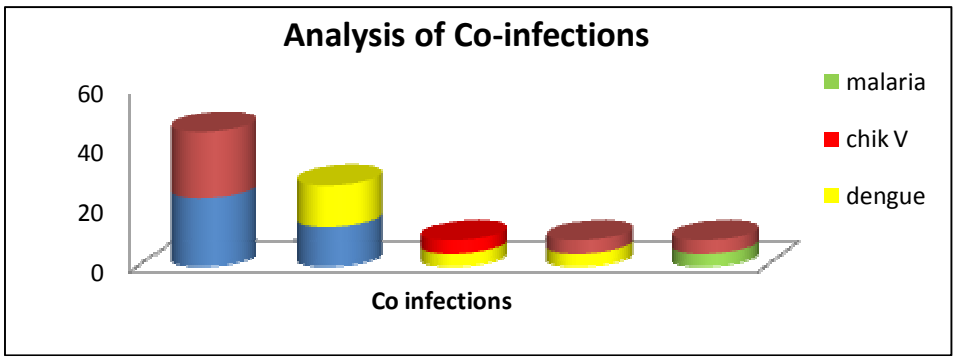
Figure 1
The etiological profile of acute undifferentiated febrile illness



Analysis of co-infections encountered among study subjects

A significant number of patients 36.7% (n= 11) with acute undifferentiated febrile illness who tested positive by one of the rapid tests had co-infections or dual infections. Among these 63.6% (n=7) were positive for Scrub typhus. The predominant group with co-infections were among those with leptospirosis and Scrub typhus 45.5% (n=5) and the next common dual infection found was among subjects with leptospirosis and dengue 27.2% (n=3). A small number 9.1% (n=1) had malaria and Scrub typhus, one had dengue and Scrub typhus and one had CHIKV and dengue (Fig.2).

Figure 2
Analysis of co-infections encountered among study subjects



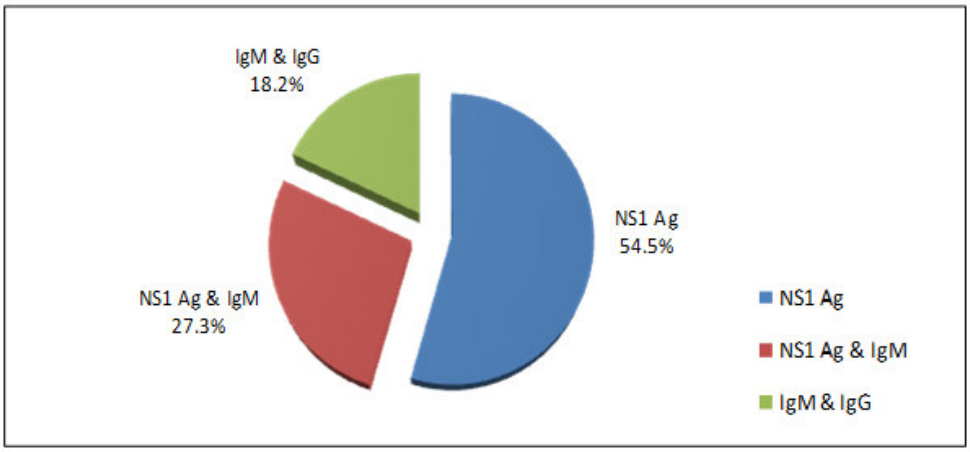
Analysis of probable predictive markers of diseases

Clinical manifestations such as maculopapular rash, overt bleeding manifestations, normal to low leukocyte counts, moderate to severe thrombocytopenia and significantly elevated hepatic transaminases were associated with dengue. Majority 81.8% (n=9) of the dengue positive study subjects presented with above mentioned clinical manifestations. Increased alkaline phosphatase, characteristic rash, lymphadenopathy, splenomegaly were associated with Scrub typhus and co-infection. Majority 71.4% (n=5) of study subjects tested positive for Scrub typhus and co-infection presented with above mentioned similar clinical presentation. Normal leukocyte counts, moderate to severe thrombocytopenia, renal failure, splenomegaly and hyperbilirubinaemia with mildly elevated serum transaminases were associated with malaria. Normal or increased total bilirubin, increased serum creatinine, head ache, lethargy, muscle pain, oliguria were associated with leptospirosis.

Rate of positivity of Dengue NS1 Ag and antibodies by rapid tests and RT-PCR

Among the rapid tests (NS1, IgM , IgG) used to detect dengue 54.5% (n=6) were only NS1 positive , 27.3% (n=3) were NS1 and IgM positive and 18.2% (n=2) were both IgM and IgG positive. The rate of positivity of NS1 Ag and NS1 with IgM; IgM and IgG is shown in Fig.3.

Figure 3
The rate of positivity of NS1 Ag and NS1 with IgM ; IgM and IgG



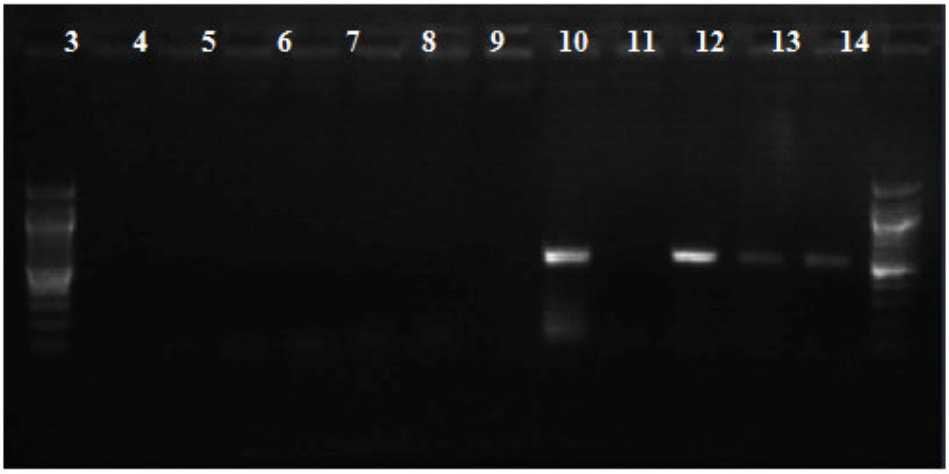
PCR – RT-PCR for Dengue

Conventional in–house RT-PCR was performed with all 30 samples for dengue. Among those tested only 2 samples were positive for dengue by RT-PCR which was also NS1 Ag positive. RT-PCR for dengue of study subjects is shown in Fig .4.

PCR – RT-PCR for CHIKV

Among the samples tested only one sample was positive for CHIKV, which had co-infection with Dengue. This patient sample had also tested positive for NS1. RT-PCR for CHIKV of study subjects is shown in Fig.5

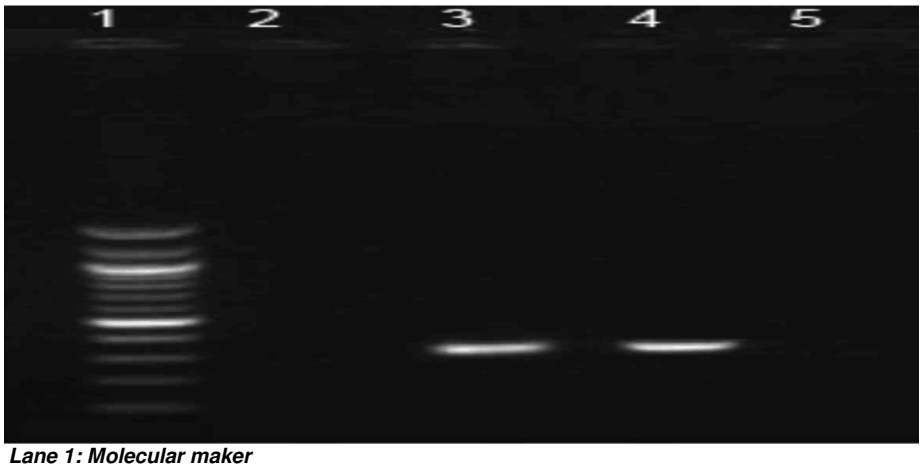
Figure 4
RT-PCR for Dengue of study subjects



Lane 1, 14: Molecular marker
Lane 2, 3, 4, 5, 6, 7, 8, 10 : Patients samples negative for 511 bp
Lane 9, 11: Patients sample positive for 511 bp

Lane 12, 13 : Positive control 1 & 2

Figure 5
RT-PCR for CHIKV of study subjects



Lane 1: Molecular maker
Lane 2: Patient sample negative for 305 bp
Lane 3: Patient sample positive for 305 bp
Lane 4: Positive control

Correlation of fever onset with rapid test positivity

We also analyzed the duration of fever positivity with any one of the rapid tests and found that 63.3% (n= 19) study subjects were detected positive within 3- 5 days of fever followed by 22.3% (n= 7) study subjects detected positive within 6 – 10 days of fever. On analyzing those tested positive for dengue we found NS1 Ag and IgM were detectable within 3-5 days of fever (Table.1)

Table 1
Correlation of onset of fever in study subjects with positivity by rapid tests

DURATION OF FEVER	NS1 Ag POSITIVE CASES	IgM & NS1Ag POSITIVE CASES	IgM & IgG POSITIVE CASES	LEPTO POSITIVE CASES	MALARIA POSITIVE CASES	CASES WITH CO – INFECTION	CHIKV PCR POSITIVE CASES	DENGUE PCR POSITIVE CASES
(NO OF POSITIVE CASES)	(6)	(3)	(2)	(3)	(2)	(11)	(1)	(2)
1-2 DAYS (1)	NIL	NIL	1	NIL	NIL	NIL	NIL	NIL
3-5 DAYS (19)	5	2	1	2	NIL	8	NIL	1
6-10 DAYS (7)	1	1	NIL	1	2	1	1	1 (ALSO NS1 Ag POSITIVE)

n=3 negative to all rapid tests including molecular tests.

Analysis of clinical diagnosis and diagnosis using rapid tests

In our study, 14 cases were clinically diagnosed as dengue, among which 13 cases were confirmed to be positive by the dengue rapid tests and only one was negative by all the rapid tests. There was one case with clinical suspicion of leptospirosis which was confirmed leptospirosis positive by the rapid ELISA, where as the other two ELISA confirmed leptospirosis cases were clinically diagnosed as viral fever.There were five cases with clinical suspicion of Malaria, among which three were confirmed positive for Malaria by the rapid tests whereas the other two cases were Leptospirosis and Scrub typhus positive by the rapid tests.

DISCUSSION

Local prevalence of individual diseases influences the prioritization of differential

diagnosis of a clinical syndrome of acute undifferentiated febrile illness. This study was mainly done to delineate the etiology of acute febrile illness in monsoon season and to review the diagnostic tools that are currently available or in development and the potential role of rapid tests in case detection, identification of prognostic markers of severe disease, surveillance and outbreak investigations. Majority 53.3% (n=16) of patients enrolled in our study with acute undifferentiated fever tested positive for dengue followed by leptospirosis and Scrub typhus. Thus in the absence of localizing signs of respiratory tract, acute undifferentiated febrile illness in monsoon months is primarily due to dengue. Co-infections of diseases such as leptospirosis with dengue or Scrub typhus also pose a problem. This has important implication in public health. All Scrub typhus detected in our study had co-infection with another agent. We are unsure if these represent true infections or a reflection of cross reactions due to any antigenic similarity between *Leptospira* and *rickettsia*. In a prospective epidemiologic study conducted in Thailand the majority (61.3%) of AUFI remained unknown with rickettsial infection, influenza and dengue fever being the most common identifiable diseases in tropical regions[6].Comparison of serology, virus isolation and RT-PCR in the diagnosis of Dengue viral infections was done in a previous study wherein virus isolation and molecular detection methods were also sensitive in the detection of the virus and its serotype, especially during days 3 to 5 of illness[6]. In our study we have not amplified the virus in cell lines prior to performing PCR. Propagation of virus in cell lines may enable detection due to amplification[3,7] This may account for only two samples testing positive for dengue by conventional RT-PCR. This discrepant result between the NS1 and RT-PCR assays may be likely due to NS1 antigen circulating in the serum for longer periods than viral RNA; thus extending the diagnostic window beyond that for RT-PCR testing.[17] In a previous study done to evaluate the performance of the rapid assays for detection of CHIKV with semi-nested RT-PCR, the sensitivity of the rapid assay was not constant and varied depending on the duration of illness[12]. Limitation of our study is that IgM tests were not performed for CHIKV.Rapid tests available for diagnosis of Leptospirosis are simple, more sensitive (96.6%) and more specific (99.79%) detects genus specific antibodies within 5-6 days of illness [10]. In our study 36.7% (n=11) of the study subjects tested positive for leptospirosis, among them 27.3% (n=3) were positive only for leptospirosis and the remaining 72.7% (n=8) had co-infection. Leptospirosis was detected earlier within 3-5 days of illnesses using IgM ELISA. There is a need to develop better diagnostic tools for Leptospirosis that are specific and avoid cross reactions. Serology is the preferred diagnostic tool for Scrub typhus. Delay in diagnosis and initiation of appropriate treatment results in severe complications such as ARDS, septic shock and multisystem organ failure culminating in death. Weil Felix test is highly specific test, but however it lacks sensitivity.Solid phase immunochromatographic assay gives a rapid, qualitative detection of IgG, IgM, IgA antibodies to *Orientia tsutsugamushi* in human serum, plasma or whole blood. In our study Scrub typhus was encountered in 23.3% (n=7). However all these patients had co-infections. In the diagnosis of Malaria, the efficacy of microscopy was compared with the rapid immunochromatographic tests and it was suggested that rapid diagnostic tests (RDTs) serve as an alternative to microscopy for early malarial diagnosis[1]. In our study there were five cases with clinical suspicion of Malaria, among which three were confirmed positive for Malaria by the rapid tests whereas the other two cases were leptospirosis and Scrub typhus positive by the rapid tests. However there is no explanation as to the exact antigenic sharing or the mechanism of the cross reaction among these acute febrile illnesses. Several studies have reported on co-infections, such as dengue and CHIKV[11,13], leptospirosis and Scrub typhus[2] , dengue and Scrub typhus, malaria and Scrub typhus , malaria and leptospirosis[14] , dengue and malaria[9]. Studies must be undertaken to develop an understanding of the immunopathogenesis of Scrub typhus,

leptospirosis, dengue and to explain the cross reaction.

CONCLUSION

In countries with an endemicity of various diseases, concurrent infections may result in illness with overlapping signs and symptoms causing substantial misdiagnosis and resulting in delay in treatment and increased mortality. Polypharmacy may lead to the development of drug resistance in micro-organisms and can be avoided in view of the etiology of AUFI. Doxycycline may be considered as a choice of empiric antibiotic to treat in AUFI in young adults. Use of rapid tests in the detection serves as a better aid in earlier diagnosis of these AUFI. Lack of knowledge of coinfection may jeopardize the health of affected patients. Our study serves as a reminder of potential coinfection and provides clues for its detection. Evaluation of tests is often performed with stored sera or evaluated against the gold standard. Actual performance of the test in field conditions is required.

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